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(REV. 5-93)

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER
2567/1F496US2

01/22/02

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

10/031893

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/19901	July 21, 2000	July 21, 1999

TITLE OF INVENTION

DROSOPHILA RECOMBINATION-ASSOCIATED PROTEIN AND METHODS FOR USE

APPLICANT(S) FOR DO/EO/US

Andrew EISEN

Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S. C. 371.

3. [] This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S. C. 371 (c) (2))
 a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
 b. [] has been transmitted by the International Bureau
 c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [] A translation of the International Application into English (35 U.S. C. 371 (c)2)).

7. [] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
 b. [] have been transmitted by the International Bureau.
 c. [] have not been made; however, the time limit for making such amendments has NOT expired.
 d. [] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).

9. [X] An unexecuted oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 (with 30 references).

12. [] An assignment document for recording. A **separate** cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.
 [] A SECOND or SUBSEQUENT preliminary amendment.

14. [] A substitute specification.

15. [] A change of power of attorney an/or address letter.

16. [] Other items or information:

EXPRESS MAIL CERTIFICATE

1/22/02 EL 677224 29 US

Date Label No.

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

D B Peck

Name (Print)

Signature

U.S. APPLICATION NO. (if known sec 37 C.F.R.1.50)	INTERNATIONAL APPLICATION NO.: PCT/US00/19901	Attorney's Docket Number 2567/1F496US2
10/031893		

17. [x] The following fees are submitted:		CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492 (a)(1)-(5)): Search Report has been prepared by the EPO [] or JPO []	**$890.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482)	**$710.00**		
No international preliminary examination fee paid to USPTO(37 CFR 4.482) but international search fee paid to USPTO (37 CFR 1.445 (a) (2)...	**$740.00**		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....	**$1,040.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)....	$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$710.00	
Surcharge of $130.00 for furnishing the oath or declaration later than []20 []30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	17-**20**		X $18.00	**$00**	
Independent Claims	**14-3**	11	X $84.00	$924.00	
Multiple dependent claims(s) (if applicable)		+280		$	
TOTAL OF ABOVE CALCULATIONS =				**$.00**	
Reduction by 1/2 for filing by small entity, if applicable.				**$.00**	
SUBTOTAL =				**$1,634.00**	
Processing fee of **$130.00** for furnishing the English translation later the [] 20 [] 39 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				**$.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$0.00	
TOTAL FEES ENCLOSED =				**$1,634.00**	
				Amount to be: refunded	$
				charged:	$

a. [X] A check in the amount of $1,634.00 to cover the above fees is enclosed.

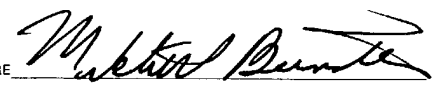
b. [] Please charge my Deposit Account No.04-0100 in the amount of $ to cover the above fees.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0100. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Darby & Darby P.C.
805 Third Avenue
New York, New York 10022-7513

SIGNATURE 

NAME MITCHELL BERNSTEIN, Ph.D.

REGISTRATION NO.46.550

10031893 072202
10/031893
JC13 Rec'd PCT/PTO 22 JAN 2002

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D B Peck — H Beck

Name (Print) Signature

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Customer No.: 07278

PATENT TRADEMARK OFFICE

Docket No: 2567/1F496-US2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Andrew EISEN

Serial No.: T.B.A.
National Phase Application of
PCT/US00/19901

Art Unit: T.B.A.

Filed: Concurrently herewith

Examiner: T.B.A.

For: **DROSOPHILA RECOMBINATION-ASSOCIATED PROTEIN AND METHODS FOR USE**

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

January 22, 2002

Sir:

Prior to examination of the above-referenced application, please amend the application as follows:

IN THE DRAWINGS

Please amend Figure 4 as submitted herewith a Letter to the Office Draftsman.

IN THE SPECIFICATION

Please insert the following paragraph to follow immediately after the title:

This application claims priority under 35 U.S.C. § 119(e) of provisional application serial no. 60/144,736, filed July 21, 1999.

Please amend the specification as follows (pursuant to rule 1.121, a marked-up version of each amended paragraph is attached in a separate document):

In the "Brief Description of the Drawings", at page 6, lines 6-8, please amend the description of Figure 4D to read as follows:

Figure 4D depicts a new motif MIVVKDESSP [SEQ ID NO: 15] shared between DRAP and other recombination-related proteins as identified through cross reactivity with anti-DRAP antibodies and sequence comparison. Depicted are motif sequences from mouse and human RAD 51, LLIVDS [SEQ ID NO: 23]; yeast RAD 51 and DCM 1, LIVVDS [SEQ ID NO: 24; E. coli REC A, VIVVDS [SEQ ID NO: 25]; FLP recombinase, MIALKDETNP [SEQ ID NO: 26]; T4 Gene 32 protein, ILVVKDPAAP [SEQ ID NO: 27], MIAVDVEMGE [SEQ ID NO: 28] and KGFSSE [SEQ ID NO: 29]; and human topoisomerase I, IKDEP [SEQ ID NO: 30], KDGSSE [SEQ ID NO: 31] and GFSSP [SEQ ID NO: 32].

In the description of Example 8 which begins on page 41, please amend the first paragraph immediately following the section heading *"Injection of DRAP and c-kit Oligos"*, which bridges pages 41-42, to read as follows:

DRAP was coinjected into male pronuclei of fertilized mouse eggs with c-kit 5'P-containing oligonucleotide, [SEQ ID NO:9] at a concentration of 10:1 molar ratio of DRAP to oligonucleotide. In this experiment approximately 500,000 molecules of DRAP and

approximately 50,000 molecules of oligonucleotide per embryo were injected using the standard transgenic protocol as described in U.S. Patent No. 4,873,191.

REMARKS

I. Drawings. Figure 4 is amended to correct typographical errors in the DRAP protein sequence given in Figure 4B. The DRAP sequence presented in Figure 4B has been amended as follows: (1) amino acids 92-93 have been changed from "IP" to "PI"amino acid; and (2) amino acid 155 is changed from "G" to "Q". The amendment is supported by the DRAP protein sequence given in Figure 2 of the application as filed. Accordingly, no new matter is added to the application by these change.

II. Specification. The specification has been amended to include reference to provisional application 60/144,736, filed July 21, 1999, as required under 37 C.F.R. § 1.78(a)(5). Applicants claim the benefit of priority of the provisional application under 35 U.S.C. 119(e).

The "Brief Description of the Drawings" has been amended to make reference to the sequences that are found in Figure 4D.

Example 8 in the specification has been amended to correct an inadvertent error. Example 8 has been amended to state, correctly, that DRAP and c-kit 5'P-containing oligonucleotide were injected into male pronuclei of fertilized mouse eggs. Support for the amendment is found in the specification at page 26, line 30 through page 27, line1. Further support is found at page 42, lines 2-3, where the specification sets forth that embryos were injected as described in U.S. Patent No. 4,873,191. Support for injection into the zygote male pronucleus is found in the '191 patent at col. 9, lines 4-8. A copy of the '191 patent is enclosed

for the Examiner's convenience. All cited patents have been incorporated into the specification in their entirety (see specification at page 8, lines 2-4).

All amendments being supported by the application as filed, no new matter has been added to the application by this Preliminary Amendment.

Applicants respectfully request a prompt and favorable action on the merits of the application.

Respectfully submitted,



Mitchell Bernstein, Ph.D.
Reg. No. 46,550
Agent for Applicants

DARBY & DARBY, P.C.
805 Third Avenue
New York, N.Y. 10022
Phone (212) 527-7700

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JC13 Rec'd PCT/PTO 22 JAN 2002

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DBPeck — Name (Print)

Signature

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Customer No.: 07278
PATENT TRADEMARK OFFICE

Docket No: 2567/1F496-US2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Andrew EISEN

Serial No.: T.B.A.
National Phase Application of
PCT/US00/19901

Art Unit: T.B.A.

Filed: Concurrently herewith

Examiner: T.B.A.

For: **DROSOPHILA RECOMBINATION-ASSOCIATED PROTEIN AND METHODS FOR USE**

MARK-UP TO AMENDMENT

IN THE SPECIFICATION

In the "Brief Description of the Drawings", at page 6, lines 6-8, the description of Figure 4D has been amended as follows:

Figure 4D depicts a new motif MIVVKDESSP [SEQ ID NO: 15] shared between DRAP and other recombination-related proteins as identified through cross reactivity with anti-DRAP antibodies and sequence comparison. Depicted are motif sequences from mouse and human RAD 51, LLIVDS [SEQ ID NO: 23]; yeast RAD 51 and DCM 1, LIVVDS [SEQ ID NO: 24; E. coli

REC A, VIVVDS [SEQ ID NO: 25]; FLP recombinase, MIALKDETNP [SEQ ID NO: 26]; T4 Gene 32 protein, ILVVKDPAAP [SEQ ID NO: 27], MIAVDVEMGE [SEQ ID NO: 28] and KGFSSE [SEQ ID NO: 29]; and human topoisomerase I, IKDEP [SEQ ID NO: 30], KDGSSE [SEQ ID NO: 31] and GFSSP [SEQ ID NO: 32].

In the description of Example 8 which begins on page 41, the first paragraph immediately following the section heading *"Injection of DRAP and c-kit Oligos"*, which bridges pages 41-42, has been amended as follows:

DRAP was coinjected into male pronuclei of fertilized mouse eggs ~~embryonic stem cells~~ with c-kit 5'P-containing oligonucleotide, [SEQ ID NO:9] at a concentration of 10:1 molar ratio of DRAP to oligonucleotide. In this experiment approximately 500,000 molecules of DRAP and approximately 50,000 molecules of oligonucleotide per embryo were injected using the standard transgenic protocol as described in U.S. Patent No. 4,873,191.

Rec'd PCT/PTO 19 JUL 2002

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Signature

Customer No.: 07278
PATENT TRADEMARK OFFICE

Docket No.: 2567/1F496-US2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Andrew EISEN

Serial No.: 10/031,893 Art Unit : TBA

Confirmation No.: 8212

Filed: January 22, 2002 Examiner: TBA

For: **DROSOPHILA RECOMBINATION-ASSOCIATED PROTEIN AND METHODS FOR USE**

July 19, 2002

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

Prior to examination, please amend the above-referenced application as follows.

IN THE CLAIMS

Please cancel claims 1-7 without prejudice or disclaimer. Claims substantially similar to claims 1-7 have been allowed in co-pending U.S. application serial number 09/621,377. By this Amendment, claims 8-19 are pending in the application.

This application is believed to be in condition for allowance. A prompt and favorable action the merits of the application is earnestly solicited.

Respectfully submitted,



Mitchell Bernstein, Ph.D.
Reg. No. 46,550
Agent for Applicants

DARBY & DARBY P.C.
Post Office Box 5257
New York, NY 10150-5257
212-527-7700

DROSOPHILA RECOMBINATION-ASSOCIATED PROTEIN AND METHODS FOR USE

FIELD OF THE INVENTION

This invention encompasses the Drosophila Recombination Associated Protein (DRAP), nucleic acid sequences encoding DRAP, and methods of using DRAP.

BACKGROUND

One common method for introducing exogenous genes in eukaryotic cells and organisms is by direct transfection. Transfection is relatively efficient but genomic integration tends to be random and regulation of gene expression can be difficult. Various strategies have been developed to enhance the control of transgene expression, limit expression to a specific tissue or link expression to a specific time during development in transgenic animals. The direct transgenic approach suffers primarily from an inability to directly modify a specific genetic locus.

Another approach for introducing exogenous genes into eukaryotic cells utilizes homologous recombination directed at a specific locus in specialized embryonic stem cells. A number of genes and gene products that participate in general and site-specific recombination in viruses, prokaryotes and eukaryotes have been identified. (Camerini-Otero, R.D., *et al.*, Ann. Rev. Genetics, 1995, 29:509-522.) These gene products and/or proteins encoded by these genes have proven useful in the molecular genetic manipulation of DNA *in vitro* and *in vivo* with applications in cloning, gene mapping and manipulation of genomes in living organisms. (Grindley, N.D., *et al.*, 1995, Cell 83: 1063-1066; and Wang, J.C., *et al.*, 1990, Cell 62: 403-406.) The use of such proteins in these applications relies upon their activity in homologous recombination.

Homologous recombination is routinely used to create "knock out" mutations in the production of mutant animals. (Nomura, T., 1997, Lab Anim. Sci. 47: 113-117; and Torres, M., 1998, Cur. Top. Dev. Biol. 36: 99-114.) This approach results in gene-directed, *i.e.* sequence directed, insertions that "knock out" gene function to produce such mutations. Although this approach is generally an inefficient process, selection methods and screening permit facilitated identification of cells bearing specific genomic modifications. The enzymatic machinery that completes this low efficiency process in specialized embryonic stem cells is unknown. It would thus be beneficial to identify one or a number of genes or gene products involved so that homologous recombination could be performed with a higher efficiency. Such site specific homologous recombination might prove useful for therapeutic purposes. The use of these specific genes and / or gene products could improve the efficiency of the knock-out process and extend the range of cell types where homologous recombination could be accomplished.

It would thus be highly desirable to develop a method for efficient, homology directed genome modification that would permit modification of genes ranging from knockouts to subtle modifications. Such methods would rely upon the use of an efficient homology dependent DNA pairing protein capable of directing a mutagenic oligonucleotide to its cognate gene within a complex genome. The aim of this approach would be to promote DNA strand exchange and force a gene conversion event that can be identified at the molecular level and is heritable. There are several, general, homology-dependent strand transferases that might be suitable for such a purpose that have been used for related purposes in vitro (*e.g.*, the RARE technique). (Ferrin, L.J. *et al.*, Nature Genetics, 1994, 6:379-383; and Ferrin, L.J., Genet. Eng., 1995, 17:21-30.

General recombinases currently used in the promotion of DNA strand transfers and gene conversions include UV Sensitive X ("UVSX") from T4 phage, Recombination Protein A ("RecA") from *E. coli* or RecA-derived peptides, or Radiation Induced Mutant 51 ("RAD51") from yeast or RAD 51 homologues from Drosophila, mouse and human. These proteins are each part of a large superfamily of recombination-related proteins. These recombinases typically require accessory proteins such as Single Strand Binding Protein ("SSB"), Replication Protein A ("RPA") and Radiation Induced

Mutant 52 ("RAD 52") in order to achieve maximal efficiency. There are also a host of site directed recombinases (of the integrase and resolvase super-families) that might be modified for such a purpose.

Drosophila embryos provide a rich source of enzymes that are involved in homologous recombination. (Eisen, A., *et al,* 1988, PNAS 85:7481-85.) It was shown that purified protein fractions possessed an efficient ATP-independent, homology-dependent strand transferase activity similar to RecA. The active fractions appeared to work catalytically as opposed to stoichiometrically. (Eisen, A. *et al.,* 1988.) The Drosophila embryonic cells are rapidly dividing and thus provide a large quantity of enzymes involved in mitotic recombination and DNA processing. A similar potent homology-dependent strand transferase activity was demonstrated to be present in nuclear extracts from Drosophila. (Eisen, A. *et al.,* 1988.) The apparent catalytic nature of this Drosophila protein activity distinguishes it from most general recombinases, typified by proteins in the RecA / Rad 51 superfamily of gene products which operate in a stoichiometric fashion (Camerini-Otero, R.D., *et al.*, 1995; and Yancey-Wrona, J.E., *et al.,* 1995, Current Biol. 5: 1149-1158; Baumann, P., *et al.,* 1996, Cell 87: 757-766; Benson, F.E., *et al.,* 1998, Nature 391: 401-404; Shinohara, A., *et al.,* 1998, Nature 391: 404-407; New, J.H., *et al.,* 1998, Nature 391: 407-410; Plasterk, R.H.A., 1993, Cell 74:781-786; and O.N. Voloshin, O.N., *et al.,* 1996, Science 272: 868-872.) The unique Drosophila homology-dependent strand transferase activity offers certain theoretical advantages for performing high efficiency gene targeting. It would thus be advantageous to utilize this Drosophila activity in the promotion of homologous recombination and homology directed gene conversion.

SUMMARY OF THE INVENTION

The present invention provides an isolated cDNA clone containing the coding region for Drosophila Recombination Associated Protein (DRAP) [SEQ ID NO: 1], a DNA encoding DRAP corresponding to nt 134-610 of the isolated cDNA clone [SEQ ID NO: 2] and the longest Open Reading Frame (ORF) contained within the isolated cDNA clone sequence which corresponds to nt 104-610 of the isolated cDNA clone [SEQ

ID NO: 3] (see Figure 1). The invention also provides an isolated DRAP polypeptide as depicted in [SEQ ID NO: 4], as well as antigenic peptides, examples of which are defined by [SEQ ID NO: 5] [SEQ ID NO: 6] and [SEQ ID NO: 7] (see Figures 2 and 4B). The invention further provides DNA vectors and transformed cells suitable for recombinant expression of DRAP.

The Drosophila Recombination Associated Protein, its homologues from other organisms or active peptides derived therefrom, as well as DNA encoding such protein are useful for homology-dependent pairing of three DNA strands. The combination of strand-transfer and topoisomerase activities associated with DRAP permits directed pairing and cleavage at defined site(s) within DNA. This in turn makes possible the isolation and/or removal of a defined segment of DNA. DRAP is also useful in cloning, genomic cloning and gene mapping.

DRAP is also useful in promoting gene disruptions or "knockout" mutations, carrying out targeted mutagenesis of specific genes and in generating transgenic animals.

Thus, in one aspect, the present invention provides methods for DNA cloning, gene isolation and gene mapping.

In another aspect, the invention provides a method for targeted mutagenesis.

In yet a further aspect, the invention provides a method for experimental and therapeutic application of DRAP driven knockouts or other modifications of genes responsible for genetic diseases and the use of DRAP driven genetic manipulation of genes in gene therapy.

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in light of the present description, claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleotide sequence of an isolated cDNA clone containing the coding region for DRAP [SEQ ID NO: 1], a DNA encoding DRAP corresponding to nt 134-610 of the cDNA clone [SEQ ID NO: 2] and the longest Open

Reading Frame (ORF) in the cDNA sequence corresponding to nt 104-610 of the cDNA clone [SEQ ID NO: 3].

Figure 2 depicts the nucleotide sequence [SEQ ID NO: 2] for DRAP, a restriction map of the nucleotide sequence and the corresponding amino acid sequence for the DRAP protein [SEQ ID NO: 4].

Figure 3A depicts a schematic outline of the purification protocol used to purify a strand transferase activity from Drosophila nuclear extracts.

Figure 3B depicts a schematic outline of the purification of fractions by elution from a single stranded (SS) DNA agarose column.

Figure 3C is a photograph of a 0.8% agarose 1 X TAE (Tris Acetate EDTA) gel demonstrating the formation of DNA aggregates (AG), nicked circles (NC) joint molecules from double stranded (DS) and single stranded circular (SSC) DNA by protein fractions retained in each of the purification steps shown in Figure 3B.

Figure 3D is a photograph of a silver-stained SDS-PAGE protein gel of the starting nuclear extract (NE) and active fractions eluted from the Sepharose (S), SS DNA (D) and Sephacryl HR-200 gel filtration (GF) columns.

Figure 3E depicts a Western blot of the purification fraction from the Sephacryl HR-200 gel filtration column described in Figure 3D and reacted separately with either a rabbit polyclonal antibody (PAb) and a neutralizing mouse monoclonal IgM (Mab).

Figure 3F depicts a silver stained 10% polyacrylamide gel of fractions eluted from an IgM immunoaffinity column loaded with Drosophila nuclear extract.

Figure 4A depicts the cDNA for the DRAP protein.

Figure 4B depicts the mature DRAP protein [SEQ ID NO:4] with the probable start site corresponding to the Met in the llth codon position of the cDNA ORF (set as amino acid #1) with the mature protein being 159 amino acids long resulting in a protein of approximately 20 kDa. The numbered shaded regions depict the three most hydrophillic antigenic peptides predicted. These peptides, numbered 1, 2 and 3 are identified as KDESSP [SEQ ID NO:5], TRRPVD [SEQ ID NO:6] and RAMARK [SEQ ID NO:7], respectively.

Figure 4C depicts the canonical DDE motifs characteristic of transposases and retroviral integrases (potential motifs). The DRAP DDE motifs closest to the canonical form are indicated below the canonical form with the locations for the D_1, D_2 and E_1 amino acids, along with the span of intervening amino acids, provided in parentheses.

Figure 4D depicts a new motif MIVVKDESSP [SEQ ID NO: 15] shared between DRAP and other recombination-related proteins as identified through cross reactivity with anti-DRAP antibodies and sequence comparison.

Figure 5A depicts *in situ* hybridization studies on embryonic Drosophila nurse cells using a digoxigenin-containing antisense DNA single stranded probe prepared from the DRAP cDNA clone. Bar equals 20 microns.

Figure 5B depicts *in situ* hybridization studies on Drosophila embryos using a digoxigenin-containing antisense DNA single stranded probe prepared from the DRAP cDNA clone. Bar equals 20 microns.

Figure 6A depicts the scheme for purification of N-terminal, His6-tagged recombinant DRAP successively purified over Ni-NTA (Qiagen), phosphocellulose (Whatman) and Sephadex 75 (Pharmacia) columns followed by centrifugal concentration (Millipore).

Figure 6B depicts a 12% polyacrylamide gel electrophoresis of purified DRAP and commercial SSB and RecA proteins (adjusted to 60 ng/ul) seen through Coomassie blue-staining

Figure 6C depicts a 0.8% 1 x TAE agarose gel electrophoretic separation of DNA species from a standard strand transfer assay for the production of joint molecules (JM) and nicked circles (NC) produced from double stranded linears (DSL) and single stranded circles (SSC) comparing RecA/SSB (Promega) to DRAP.

Figure 6D depicts a 0.8% agarose 1 x TAE gel electrophoresis of DNA species resulting from a topoisomerase assay utilizing a plasmid preparation containing both supercoiled (SC) and nicked circular (NC) forms incubated with DRAP (60 ng) or RecA/SSB (240/200 ng) at 37°C for 30 min.

Figure 6E depicts a 0.8% agarose 1 x TAE gel electrophoresis demonstrating that the recombinant DRAP will form large protein-DNA aggregates (AG) with SSC and DSL substrates in a strand transferase assay.

Figure 6F depicts a 0.8% 1 x TAE gel electrophoresis of DNA species produced from a recombinase reaction using an end labeled DSL substrate.

Figure 7 depicts an ATP-dependent three strand reaction demonstrating that DRAP produces displaced strands and does not possess helicase activity. DSL is defined as double stranded linear DNA, SSC is defined as single stranded circular DNA and SCP is defined as supercoiled plasmid double stranded DNA.

Figure 8 depicts a polyacrylamide gel demonstrating that DRAP does not possess nuclease activity as tested with end labeled oligonucleotides.

Figure 9 depicts a model of DRAP driven molecular DNA reactions.

Figure 10 depicts a diagram of a model for gene conversion driven by a recombinase and an oligonucleotide.

Figure 11 depicts the results of preliminary studies performed with 5'OH-containing oligonucleotides.

Figure 12 depicts the murine c-kit Exon 17 CDNA sequence [SEQ ID NO: 8], the potential mutagenic oligonucleotide ("oligo") for the locus (underlined portion), namely, tgtattcacagagatttggcagccaggaata [SEQ ID NO: 9], PCR primers to be used in Restriction Fragment Length Polymorphism ("RFLP") analysis (see double arrows), namely ggacagtgtattcac [SEQ ID NO: 10] and ttgcgatttcgggctag [SEQ ID NO: 11], the DNA nucleotide sequence of the W42 mutation [SEQ ID NO:12], and the amino acid protein sequences corresponding to SEQ ID NO: 8 and 12, namely [SEQ ID NO:13] and [SEQ ID NO:14], respectively. The figure also provides restriction maps of the DNA sequences.

Figure 13 depicts a photograph of a white spotted mouse produced from the co-injection of DRAP and the mutagenic c-kit oligonucleotide and a photograph of an unspotted control mouse.

DETAILED DESCRIPTION OF THE INVENTION

All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of conflict, the present description, including definitions, will control.

This invention is directed to an isolated nucleic acid which encodes a recombination-associated protein found in *Drosophila melanogaster* embryos, Drosophila Recombination Associated Protein (DRAP). The invention is also directed to the DRAP protein which exhibits both recombinase (homology-dependent strand transferase) and topoisomerase activity. This combination of properties provides for a protein useful in a number of activities including cloning or gene targeting protocols.

Definitions:

"Recombinase activity" as used herein refers to the promotion of homologous pairing and DNA strand exchange. Recombinases can be site-specific or general and can operate in a variety of biological contexts by a variety of biochemical mechanisms. Recombinase activity can also be promoted by smaller peptides derived from either native recombinase proteins or mutagenized peptides derived therefrom.

"Topoisomerase activity" as used herein refers to the ability of a protein to change the linking number of DNA. The "linking number" as used herein refers to the number of times the two strands of a closed DNA duplex cross over each other.

"Nucleic acid" or "polynucleotide" as used herein refer to purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, *i.e.*, DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases.

An "isolated" polypeptide or nucleic acid is defined as one that is unaccompanied by at least some of the material with which it is associated in its natural state. Generally, an isolated polypeptide constitutes at least about 1%, preferably at least

about 10%, and more preferably at least about 50% by weight of the total protein in a given sample. Included in the polypeptide weight are alternative forms such as differentially glycosylated or phosphorylated or otherwise post-translationally modified forms. An "isolated" nucleic acid sequence is present as other than a naturally occurring chromosome or transcript in its natural state and typically is removed from at least some of the proteins with which it is normally associated on a natural chromosome. A "partially pure" nucleotide sequence constitutes at least about 5%, preferably at least about 30%, and more preferably at least about 90% by weight of total nucleic acid present in a given fraction.

Also encompassed by the invention are nucleic acids that are hybridizable to, or derived from, the DRAP sequences described above.

A nucleic acid or polypeptide sequence that is "derived from" a designated sequence refers to a sequence that is related in nucleotide or amino acid sequence to a region of the designated sequence. For nucleic acid sequences, this encompasses sequences that are homologous or complementary to the sequence, as well as "sequence-conservative variants" and "function-conservative variants." For polypeptide sequences, this encompasses "function-conservative variants." Sequence-conservative variants are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Function-conservative variants are those in which a given amino acid residue in a polypeptide has been changed without altering the overall conformation and function of the native polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). "Function-conservative" variants of a designated polypeptide also include any polypeptides that have the ability to elicit antibodies specific to the designated polypeptide.

Nucleic acids are "hybridizable" to each other when at least one strand of nucleic acid can anneal to another nucleic acid strand under defined stringency conditions. Stringency of hybridization is determined, *e.g.*, by a) the temperature at which hybridization and/or washing is performed, and b) the ionic strength and polarity (*e.g.*, formamide concentration) of the hybridization and washing solutions, as well as other

parameters. Hybridization requires that the two nucleic acids contain substantially complementary sequences; depending on the stringency of hybridization, however, mismatches may be tolerated. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementarity, variables well known in the art.

In one embodiment, the invention relates to isolated nucleic acids capable of hybridizing with the DRAP sequences above or with their complements under high stringency hybridization conditions, an example of which is defined below.

-- Prehybridization treatment of the support (*e.g.* nitrocellulose filter or nylon membrane), to which is bound the nucleic acid capable of hybridizing with that of *D. melanogaster* DRAP, at 65°C for 6 hours with a solution having the following composition: 4 x SSC, 10 x Denhardt (1X Denhardt is 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA (bovine serum albumin); 1 x SSC consists of 0.15M of NaCl and 0.015M of sodium citrate, pH 7);

-- Replacement of the pre-hybridization solution in contact with the support by a buffer solution having the following composition: 4 x SSC, 1 x Denhardt, 25 mM NaPO4, pH 7, 2 mM EDTA, 0.5% SDS, 100 µg/ml of sonicated salmon sperm DNA containing a nucleic acid derived from the sequence of the DRAP as probe, in particular a radioactive probe, and previously denatured by a treatment at 100°C for 3 minutes;

-- Incubation for 12 hours at 65°C;

-- Successive washings with the following solutions: (i) four washings with 2 x SSC, 1 x Denhardt, 0.5% SDS for 45 minutes at 65°C; (ii) two washings with 0.2 x SSC, 0.1 x SSC for 45 minutes at 65°C; and (iii) 0.1 x SSC, 0.1% SDS for 45 minutes at 65°C.

The invention also encompasses any nucleic acid exhibiting the property of hybridizing specifically with the above-described *D. melanogaster* DRAP under the conditions described above, but at approximately 40°C, including successive washings in 2X SSC at 45°C for 15 minutes.

It will be understood that the conditions of hybridization defined above constitute preferred conditions for the hybridization, but are in no way limiting and may be

modified without in any way affecting the properties of recognition and hybridization of the probes and nucleic acids mentioned above.

The salt conditions and temperature during the hybridization and the washing of the membranes can be modified in the sense of a greater or lesser stringency without the detection of the hybridization being affected. For example, it is possible to add formamide in order to lower the temperature during hybridization.

Nucleic acids that hybridize to the DRAP sequences of the invention may be of any length. In one embodiment, such polynucleotides are at least 8-25, preferably at least 100 and most preferably at least 200 nucleotides long. In another embodiment, the polynucleotide that hybridizes to the polynucleotide of the invention is of the same length as the polynucleotide of the invention.

"Functional homology" to DRAP polypeptide is defined by one or more biochemical properties specific to DRAP that are shared. Examples of such properties include recombinase and topoisomerase activity, and demonstration of antigenicity using anti-DRAP antibodies.

"Non-homogeneous" as used herein is defined as a fraction which is not purified to homogenity.

DRAP-Encoding Nucleic Acids and Polypeptides

The present invention encompasses nucleic acid sequences from *D. melanogaster* that encode for DRAP as depicted in Figure 1, *e.g.* the nucleotide sequence of an isolated cDNA clone containing the coding region for DRAP [SEQ ID NO: 1], a DNA encoding DRAP corresponding to nt 134-610 of the cDNA clone [SEQ ID NO: 2] and the longest Open Reading Frame (ORF) in the cDNA sequence corresponding to nt 104-610 of the cDNA clone [SEQ ID NO: 3]. Methods used for determining the relevant nucleic acid sequences are described in Example 1 below, and the deduced DRAP amino acid sequence, *i.e.* the gene encoding the DRAP polypeptide from *D. melanogaster* [SEQ ID NO: 4], of approximately 20 kDa is shown in Figures 2 and 4B.

The present invention encompasses DNA and RNA sequences, and sense and antisense sequences. DRAP-encoding sequences according to the present invention

may be modified by transitions, transversions, deletions, insertions, or other modifications such as alternative splicing. The invention also encompasses genomic DRAP sequences and DRAP gene flanking sequences, including DRAP regulatory sequences. Nucleic acid sequences encoding DRAP polypeptides may also be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. Other useful heterologous sequences are known to those skilled in the art. Furthermore, the nucleic acids can be modified to alter stability, solubility, binding affinity and specificity. For example, DRAP encoding sequences can be selectively methylated. The nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

In general, nucleic acid manipulations according to the present invention use methods that are well known in the art, as disclosed in e.g. *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor; or *Current Protocols in Molecular Biology*, Eds. Aufubel, Brent, Kingston, More, Freidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, NY, 1992 and regularly updated versions.

The invention also encompasses any hybridizable nucleic acid exhibiting the property of hybridizing specifically with the above-described DRAP-encoding DNA under the hybridization conditions described above, but at 40°C, including successive washings in 2X SSC at 45°C for 15 minutes.

It will be understood that the conditions of high stringency hybridization defined above constitute preferred conditions for hybridization, but are in no way limiting and may be modified in ways known in the art which do not affect the overall properties of recognition and hybridization of the probes and nucleic acids mentioned above.

The invention also encompasses vectors comprising DRAP-encoding nucleotide sequences, cells comprising the vectors, and methods for producing DRAP that involve culturing the cells.

A large number of vectors, including plasmid and fungal vectors, have been

described for expression in a variety of eukaryotic and prokaryotic hosts. Such vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression cassettes. The inserted DRAP coding sequences may be synthesized, isolated from natural sources, prepared as hybrids, etc. Ligation of the coding sequences to the transcriptional regulatory sequences may be achieved by known methods. Suitable host cells may be transformed/transfected/infected by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, fungal infection, microinjection, microprojectile, or other established methods known in the art.

A wide variety of host/expression vector combinations may be employed in expressing DNA sequences encoding DRAP. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids col El, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, Gene 67:31-40, 1988), pMB9 and their derivatives, plasmids such as RP4; phage DNAs, *e.g.*, the numerous derivatives of phage l, *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 micron plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Appropriate host cells for expressing protein include bacteria, Archaebacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. Of particular interest are *E. coli, B. subtilis, S. cerevisiae, Sf9* cells, C129 cells, 293 cells, *Neurospora*, and CHO cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in the art.

Under the appropriate expression conditions, host cells can be used as a source of recombinantly produced DRAP.

Advantageously, vectors may also include a promoter sequence operably linked to the DRAP encoding portion. The encoded DRAP may be expressed by using any suitable vectors and host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. The particular choice of vector/host is not critical to the invention.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

Expression of DRAP or DRAP fragments may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control DRAP gene expression include, but are not limited to, Cytomegalovirus ("CMV") immediate early promoter (CMV promoter; US Patent Nos. 5,385,839 and 5,168,062) the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290: 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22: 787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296: 39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, *et al.*, 1978, Proc. Natl. Acad. Sci. U.S.A. 75: 3727-3731), or the *tac* promoter (DeBoer, *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80: 21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242: 74-94; promoter elements from yeast or other fungi such as the Gal

4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38: 639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50: 399-409; MacDonald, 1987, Hepatology 7: 425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315: 115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38: 647-658; Adames *et al.*, 1985, Nature 318: 533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7: 1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45: 485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1: 268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5: 1639-1648; Hammer *et al.*, 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1: 161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315: 338-340; Kollias *et al.*, 1986, Cell 46: 89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48: 703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314: 283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234: 1372-1378).

Nucleic acids encoding wild-type or variant DRAP polypeptides may also be introduced into cells by recombination events. For example, such a sequence can be introduced into a cell, and thereby effect homologous recombination at the site of an endogenous gene or a sequence with substantial identity to the gene. Other recombination-based methods, such as non-homologous recombinations or deletion of endogenous genes by homologous recombination, may also be used.

The invention also encompasses isolated and purified DRAP polypeptides, including, *e.g.*, a polypeptide having the amino acid sequence depicted in Figures 2 and 4B

[SEQ ID NO: 4] as well as function-conservative variants of this polypeptide, including fragments that retain recombinase and/or topoisomerase activity as described above, and antigenic DRAP peptides, examples of which have the amino acid sequence depicted in Figure 4B [SEQ ID NO: 5], [SEQ ID NO: 6] and [SEQ ID NO: 7].

DRAP-derived polypeptides according to the present invention, including function-conservative variants, may be isolated from wild-type or mutant *D. melanogaster* cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which a DRAP-derived protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion proteins. Alternatively, polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis.

The DRAP amino acid sequence [SEQ ID NO: 4] possesses a number of potential sites for post-translational modification of the encoded protein as determined by using the Prosite® analysis software program (ExpasY). These post-translational modification sites may be important for biological function of the recombinase and include the following:

1) N-glycosylation site beginning at amino acid number 4 is defined by the amino acid sequence NNSS.

2) cAMP- and cGMP-dependent protein kinase phosphorylation site beginning at amino acid number 137 is defined by the amino acid sequence RKLT.

3) Protein kinase C phosphorylation sites beginning at amino acid numbers 23, 43 and 81 are defined by the amino acid sequences TFK, TRR and SPK, respectively.

4) Casein kinase II phosphorylation site beginning at amino acid number 7 is defined by the amino acid sequence STTD.

5) N-myristylation sites beginning at amino acid numbers 30, 39, 52 and 149 are defined by the amino acid sequences GSEVAR [SEQ ID NO: 16], GIKYTR [SEQ ID NO: 17], GVAKNL [SEQ ID NO: 18] and GLLATG [SEQ ID NO: 19], respectively.

DRAP has no close homologues but shares a short motif identified as MIVVKDESSP [SEQ ID NO:15] and shown in Figure 4D, which is related to a motif found in several general recombinases and other recombination-associated proteins (Camerini-Otero, R.D., *et al.*, 1995; and Heyer, W.D., 1994, Experientia 50: 223-233.). DRAP also contains several DDE motifs. (*See* Figures 4B and 4C.) The DDE motif is found in a number of transposases and retroviral integrases. (Bushman, F.D., *et al.*, 1993, Proc. Nat. Acad. Sci. 90: 34283432; Cheng, C., *et al.*,1998, Cell 92: 841-850; Gangloff, S., *et al.*,1994, Experientia 50: 261-269; Rice, P. *et al.*, 1995, Cell 82: 209-220; and Sherratt, D.J., *et al.*, 1998, Cell 93: 149-152.) Three amino acids, namely, D, D and E, have been shown to be involved in catalysis and are found in otherwise unrelated proteins. The D, D and E amino acids in the motif are separated by a variable number of amino acids as shown in Figure 4B.

"Purification" of DRAP refers to the isolation of the polypeptide in a form that allows its recombinase/topoisomerase activity to be measured without interference by other components of the cell in which the polypeptide is expressed. Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine ("His6") sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against DRAP or against peptides derived therefrom can be used as purification reagents. Other purification methods known in the art are possible.

Proteins that interact strongly with DRAP can be used in a variety of

methods to (affinity) purify DRAP, enhance or inhibit DRAP activity or substitute for immunoaffinity reagents (antibodies) in the detection of DRAP or DRAP homologues. DRAP-interacting proteins can identified by a variety of techniques such as co-immunoprecipitation, co-purification or by genetic techniques such as that utilized in the yeast two-hybrid method (Drees, B.L, Curr Opinions Chem Biol. 3: (1999) 64-70), functional screening of expression libraries with DRAP and variations thereof.

The isolated polypeptides may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. They may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

In a standard strand transfer assay (*see* Figures 3C, 6C and 7; and Examples 1 and 6 below), homologous M13 phage double stranded linear and single stranded circular DNAs were incubated together for various times, deproteinized with SDS and followed by agarose gel electrophoresis. Highly purified, but non-homogeneous, native protein fractions have a potent strand transferase activity (Eisen, *et. al.*, 1988.) and yield bands correlated with joint molecules and/or nicked circles.

The non-homogeneous fractions were enriched for a 20kDa species that was immunoreactive with neutralizing IgM monoclonal antibody when run on a polyacrylamide gel. (Figure 3D, fraction 28.) Most forms of bacterially expressed recombinant DRAP protein exhibited a weak strand transferase activity when ethidium bromide-stained agarose gels were examined for the appearance of joint molecules or nicked circles.

Initially, the reason for the apparently poor strand transferase activity exhibited by the recombinant protein was not clear. It was determined that the absence of joint molecules and nicked circles was due to the presence of another recombination-related activity exhibited by the recombinant DRAP protein, namely topoisomerase activity.

Topoisomerase activity is an important feature of general homologous recombination utilized to resolve joint molecules formed by strand transferase activity. (Gangloff, S., *et al.*, 1994; Dunderdale, H.J., *et al.*, 1991, Nature 354: 506-510; and

Eggleston, A.K., *et al.*, 1997, Cell 89: 607-617.) This activity is not a feature of the known general recombinases of the RecA/Rad51 family or their active peptides. However, as noted above, topoisomerase activity has been shown to be an integral property of the prokaryotic site-specific recombinases, transposases and viral integrases that share the DDE motif. Because DRAP contains several potential DDE motifs, it was examined for topoisomerase activity.

The DRAP recombinant protein, as defined by [SEQ ID NO:4] exhibits a potent, ATP independent, topoisomerase-like activity. Incubation of the protein with a mixed supercoiled /nicked circular substrate lead to the relaxation and subsequent linearization of the substrates (*See* Example 6 and Figure 6D). Because nicked circles are the end product of a completed strand transfer reaction in the assay with M13 DNA, it was postulated that these products were further converted by the DRAP protein topoisomerase activity to a new double stranded linear molecule. Thus, in the standard strand transfer assay with M13 viral DNA substrate, the DRAP protein demonstrated both strand transferase and topoisomerase activities. Nicked circular products that formed became linearized and, in this case, the displaced linear single strand migrated similarly to the single strand circle, and made it appear that there was little or no strand transferase activity on ethidium-stained agarose gels. Strand transfer can be shown, however, by endlabeling the duplex substrate (*see* Figures 6E and 6F).

In the end labeling experiment, where both ends of the duplex were end-labeled with ^{32}P, the fate of both strands of the duplex was observed. In a reaction that required both single strand and double strand DNA as well as ATP and magnesium, one strand was displaced from the duplex substrate by the invading single stranded circular substrate. The nicked circle that forms is converted to a linear duplex identical in length to the starting substrate. The displaced linear single stranded DNA migrated slightly behind the single stranded circular substrate. Complete strand transfer occurred and the nicked circular intermediate product was linearized (Figure 9). The examples discussed here and provided below demonstrate that DRAP carries out both strand transfer and topoisomerase activity.

Anti-DRAP Antibodies

The present invention also encompasses antibodies that are specific for DRAP or fragments of DRAP as described above, including, but not limited to, antibodies generated against peptides defined by [SEQ ID NO: 5], SEQ ID NO: 6] and [SEQ ID NO:7]. As used herein, antibodies "specific" for DRAP include, without limitation, antibodies that: bind to DRAP but do not bind to other nuclear proteins bind Rec-A-like proteins with DDE motifs from non-Drosophila species with a lower affinity than to DRAP; identify associational or other functional domains present in DRAP but not in other species, and the like. The antibodies may be polyclonal or monoclonal. The antibodies may be elicited in an animal host by immunization with DRAP or fragments derived therefrom or may be formed by *in vitro* immunization of immune cells. The immunogens used to elicit the antibodies may be isolated from *D. melanogaster* cells or produced in recombinant systems.

The antibodies may also be produced in recombinant systems programmed with appropriate antibody-encoding DNA. Alternatively, the antibodies may be constructed by biochemical reconstitution of purified heavy and light chains. The antibodies include hybrid antibodies (*i.e.*, containing two sets of heavy chain/light chain combinations, each of which recognizes a different antigen), chimeric antibodies (*i.e.*, in which either the heavy chains, light chains, or both, are fusion proteins), and univalent antibodies (*i.e.*, comprised of a heavy chain/light chain complex bound to the constant region of a second heavy chain). Also included are Fab fragments, including Fab' and $F(ab)_2$ fragments of antibodies.

Methods for the production of all of the above types of antibodies and derivatives are well-known in the art and are discussed in more detail below. For example, techniques for producing and processing polyclonal antisera are disclosed in Mayer and Walker, 1987, *Immunochemical Methods in Cell and Molecular Biology,* (Academic Press, London). Such antibodies are conveniently made using the methods and compositions disclosed in Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, as well as immunological and hybridoma technologies known to those of ordinary skill in the art. Where natural or synthetic DRAP-derived

peptides are used to induce a DRAP-specific immune response, the peptides may be conveniently coupled to a suitable carrier such as KLH and administered in a suitable adjuvant such as Freunds. Preferably, selected peptides are coupled to a lysine core carrier substantially according to the methods of Tam (Proc. Natl. Acad. Sci. USA 85:5409, 1988).

In one embodiment, purified recombinant DRAP is used to immunize mice, after which their spleens are removed, and splenocytes used to form cell hybrids with myeloma cells and obtain clones of antibody-secreted cells using techniques that are standard in the art. The resulting monoclonal antibodies can be screened using *in vitro* assays such as those described above for binding to DRAP.

Anti-DRAP antibodies may be used to quantify DRAP, using immunoassays such as, but not limited to ELISA. Anti-DRAP antibodies may also be used to identify, isolate, and purify DRAP or related proteins from different sources, and to perform subcellular and histochemical localization studies.

Potential Applications For DRAP

Overview:

The Drosophila Recombination Associated Protein, its homologues from other organisms or active peptides derived therefrom, are useful in any situation in which homology-dependent pairing of three DNA strands is desired. DRAP may be used to pair a single stranded probe or nucleic acid (RNA, DNA, PNA or other DNA compatible chemically-derived purine/pyrimidine base-pairing oligonucleotide) having a native or mutant sequence to homologous regions in any duplex DNA such as genomic DNA, isolated linear DNA or cloned DNA *in vivo* or *in vitro*. In addition to its use with single stranded nucleic acids DRAP may be used with any duplex DNAs and/or duplex RNAs which possess single stranded extensions at either the 5' or 3' ends. Basic methods for carrying out such homology-dependent pairings are as follows: pre-incubation (complexing) of DRAP and the specific single-stranded probe(s) (or double stranded with single stranded ends) followed by (i) addition of the complexed material to a duplex DNA, *in vivo* or *in vitro*, and (ii) incubation. Upon incubation, the probe-DRAP mixture would

enzymatically modify the duplex DNA. A diagram depicting gene conversion events driven by a recombinase and an oligonucleotide is provided in Figure 10.

The additions *in vivo* are carried out according to any art recognized method including but not limited to any one of the commonly used transformation, transfection, electroporation, microinjection, or ballistic methods that use chemical, physical or biological means to introduce DNA and protein into isolated cells or the cells of specific tissues or organs and/or their DNA and RNA-containing compartments and organelles such as the mitochondria and nuclei. (*See, Current Protocols in Molecular Biology* 1992 and regularly updated versions.

The combination of strand-transfer and topoisomerase (cleavage) DRAP activities allows for directed pairing and cleavage at defined site(s) within DNA. This in turn permits the isolation and/or removal of a defined segment of DNA. DRAP could be used alone or in combination with other DNA-modifying enzymes and/or proteins to effect subsequent manipulation of the target duplex DNA.

DRAP can also be used to replace RecA (+/- SSB), RAD 51 (+/- RAD 52) or their homologues and biochemically active peptides in a variety of current methods, *e.g.* the RARE method. (Ferrin, L.J. *et al.,* 1994; and Ferrin, L.J., 1995.) This would result in an improvement in the efficiency of the reactions involved because DRAP acts catalytically rather than stoichiometrically. In other words, only a single step is involved in the reaction because DRAP contains both strand transferase and topoisomerase activity. (See Example 6 below.)

DRAP can also be modified, at sites that do not diminish its biochemical activities utilizing strand transfer or topoisomerase assays, to include peptide sequences that would direct and/or enhance the delivery of the protein to specific organs or cellular compartments and organelles. One non-limiting example of a system to be used in such directed delivery would include liposomes with organ specific uptake ligands incorporated on them with DRAP and mutagenic DNA encapsulated inside such liposomes. Likewise, the native sequence could be modified by random or directed mutagenesis, resulting in amino acid substitutions, that could be screened and selected for enhancement in the desired biochemical activities of the mutant proteins.

Some of the specific applications for DRAP are noted below.

Cloning:

DRAP can act as a "universal" restriction enzyme. A single defined oligonucleotide or pair of oligonucleotides is used to make a defined, single-step cleavage or cleavages in a template duplex DNA. As used herein the term "universal" refers to the ability of DRAP to direct cleavage to any user-specified sequence, the sequence to be cleaved being determined by the homology of the single-stranded (or double stranded with single stranded ends) probe selected to be paired with the duplex DNA to be cleaved in the presence of DRAP. As used herein the term "template duplex DNA" is defined as any double stranded DNA in a genome, plasmid, linear fragment or the like. A pair of such single-step cleavages would lead to a deletion of a defined fragment from the duplex DNA template. Pairing, initiated by defined oligonucleotide sequence(s) and DRAP protein would determine the user-defined cleavage site(s). Examples of such duplex DNA are genomic DNA, isolated linear fragments or DNA contained within any variety of cloning vehicles such as HACs (human artificial chromosomes), YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes) and PACs (phage artificial chromosomes), cosmids, plasmids, phagemids, viruses or other vectors.

One method for carrying out such single-step cleavage(s) reaction includes but is not limited to incubation of a defined oligonucleotide(s), DRAP and duplex DNA in a buffer containing NaCl, Mg++ and other components optimized for pairing and cleavage at a temperature and pH suitable for the target duplex DNA as is well known in the art and as is described in the Examples below for either the strand transferase or topoisomerase activity assays. As shown below, the reactions initiated by DRAP lead to the homologous recombination of a gene. Such recombination leads to the cutting out of the native DNA. Small oligonucleotides homologous to the gene of interest would lead to the removal of other pieces of the native genomic DNA. The cut DNA fragment or fragments could then be isolated and subjected to further art recognized manipulations in order to clone the desired DNA fragment.

Genomic Cloning & Mapping:

The isolation of a defined duplex DNA fragment by sequence-directed cleavage would provide for the cloning of a large gene or gene fragment from genomic DNA when only the complete or partial cDNA sequence is known. Oligonucleotides from the 5' and 3' portion of the known cDNA sequence along with DRAP would be used to cut out a large gene fragment from genomic DNA. The gene fragment could be isolated from the larger high molecular weight genomic DNA by pulse-field gel electrophoresis or other methods. This large genomic fragment could then be used as a high quality probe for genomic library screening or *in situ* mapping techniques. The ability to make defined cleavages and deletions in duplex DNA, even within the complexity of a whole genome, serves as the basis for genetic modifications of living cells and organisms with DRAP.

DRAP - Promoted Gene Disruption or "Knockout" and Targeted Mutagenesis:

DRAP - promoted gene disruption can be carried out in order to generate knock out mutations and to evaluate the effects of such knock outs on the cell and/or animal in which they are generated. It is also possible to evaluate the effect of compounds or disease-related genes in "knockout" animals or cells, *e.g.*, to identify a compound that can compensate for a defect in disrupted gene activity or to determine the effect a particularly knockout has on progression and/or occurrence of disease. This technology permits manipulation of single units of genetic information in their natural position in a cellular genome and to examine the results of that manipulation in the background of a terminally differentiated organism.

A "knockout mammal" is a mammal (*e.g.*, mouse, cat, dog, cow, sheep, goat, etc.) that contains within its genome a specific gene that has been inactivated by the method of gene targeting (*see, e.g.*, US Patents No. 5,777,195 and No. 5,616,491). A knockout mammal includes both a heterozygous knockout (*i.e.*, one defective allele and one wild-type allele) and a homozygous mutant (*i.e.*, two defective alleles). Preparation of a knockout mammal requires first introducing a nucleic acid construct that will be used to suppress or disrupt expression of a particular gene into an undifferentiated cell type termed an embryonic stem cell. This cell is then injected into a mammalian embryo. A

mammalian embryo with an integrated cell is then implanted into a foster mother for the duration of gestation. Zhou, *et al.* (Genes and Development, 9:2623-34, 1995) describes PPCA knock-out mice.

The term "knockout" refers to partial or complete suppression of the expression of at least a portion of a protein encoded by an endogenous DNA sequence in a cell. The term "knockout construct" refers to a nucleic acid sequence that is designed to decrease or suppress expression of a protein encoded by endogenous DNA sequences in a cell. The nucleic acid sequence used as the knockout construct is typically comprised of (1) DNA from some portion of the gene (exon sequence, intron sequence, and/or promoter sequence) to be suppressed and (2) a marker sequence used to detect the presence of the knockout construct in the cell. When the suppression or knockout of a given gene will result in a known and visible phenotype, a marker gene is not required.

Typically, the knockout construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to prevent or interrupt transcription of the native DNA sequence. Such insertion usually occurs by homologous recombination (*i.e.*, regions of the knockout construct that are homologous to endogenous DNA sequences hybridize to each other when the knockout construct is inserted into the cell and recombine so that the knockout construct is incorporated into the corresponding position of the endogenous DNA). The knockout construct nucleic acid sequence may comprise 1) a full or partial sequence of one or more exons and/or introns of the gene to be suppressed, 2) a full or partial promoter sequence of the gene to be suppressed, or 3) combinations thereof. Typically, the knockout construct is inserted into an embryonic stem cell (ES cell) and is integrated into the ES cell genomic DNA, usually by the process of homologous recombination. This ES cell is then injected into, and integrates with, the developing embryo.

The phrases "disruption of the gene" and "gene disruption" refer to insertion of a nucleic acid sequence into one region of the native DNA sequence (usually one or more exons) and/or the promoter region of a gene so as to decrease or prevent expression of that gene in the cell as compared to the wild-type or naturally occurring sequence of the gene. When this nucleic acid construct is then transfected into a cell, the construct will

integrate into the genomic DNA. Thus, many progeny of the cell will no longer express the gene at least in some cells, or will express it at a decreased level, as the DNA is now disrupted by the construct DNA. In addition, constructs with mutations which result in a change in the coding or non-coding portion of the gene that produces a non-functioning gene product could also be used. Examples of such constructs include, but are not limited to, those constructs containing deletions, inversions, transitions, transversions and alterations in splice donor/acceptor sites.

Previously, homologous recombination carried out in the generation of knockout mutations required the DNA knock out construct to be at least about 1 kilobase (kb) in length and preferably 3-4 kb in length, thereby providing sufficient complementary sequence for recombination upon introduction of the knockout construct into the genomic DNA of the cell. DRAP can facilitate such homologous recombination driven manipulations with large constructs. However, in contrast to the currently practiced methods encompassing complicated and lengthy procedures to establish and generate knockout mice, DRAP provides for the ability to insert into or modify genomic DNA directly *in vivo* using "oligonucleotides", *i.e.* less than about 100 bp, by less cumbersome transfection methods. DRAP thus increases the efficiency of the production of transgenic animals.

A mammal in which a gene has been homologously recombined in the manner described above is hereby defined as a transgenic mammal.

The DRAP protein of the invention thus provides for the targeting of a specific mutagenic oligonucleotide or oligonucloetides to one or more cognate gene or genes in a complex genome. As used herein a "mutagenic oligonucleotide" means a DNA sequence that contains a mutation of change within the corresponding endogenous gene sequence. Non-limiting examples of oligonucleotides to be used include those with one or more base substitutions, deletions or insertions in such cognate gene, intron, exon or regulatory element.

The DRAP protein, in conjunction with endogenous DNA repair and recombination proteins known to those skilled in the art, effects a direct modification of the targeted locus. Direct co-injection of protein with mutagenic oligonucleotides into the

pronucleus of fertilized mouse eggs results in viable transgenic mammals.

As shown in Examples 7 and 8 below, recombinant DRAP was coinjected with several mutagenic oligonucleotides to induce gene conversion events in transgenic mice producing targeted mutations in the genome of such mice. Microinjected DRAP and a mutagenic oligonucleotide target a specific gene in the genome of a mouse zygote and modify the locus so that the gene's function is ablated (knocked-out). The protocol may use a single oligonucleotide to initiate a single cleavage. That cleavage is then repaired /modified by the double strand DNA break repair enzymes of the cell. When the function of the encoded gene is disturbed by this cleavage/repair process the gene is knocked-out with a frequency of up to about 30 %. This direct knock-out approach is considerably easier and less expensive to perform than the methods currently in practice.

This method could be further enhanced by the use of two mutagenic oligonucleotides from the gene of interest, as was suggested above for the creation of a deletion to be used in cloning and gene mapping applications. This would produce an interstitial deletion in the gene that is more difficult for a cell to repair than a single double strand break and would thus further enhance the likelihood of ablating gene function.

These methods are generally applicable to all cell types and are not limited to the generation of transgenic animals. One or more native or mutant oligonucleotides and DRAP can also be introduced into a particular cell or organ by any of the known methods described above and known to those skilled in the art. Such an insertion leads to a knockout in a specific cell or organ type and would be useful to ablate function of a disease-causing gene and/or fusion gene in the treatment of metabolic disorders or cancerous states.

DRAP cDNA could also be introduced, first or later, with the specific oligonucleotide, as an episomal genetic element or as a transgene under the control of an appropriately designed expression system that would allow for tissue specific and/or induction agent controlled expression. Such tissue specificity could be achieved via controlled expression of DRAP using a tissue-specific promoter or an inducible promoter. This method also permits targeted modification of specific DNA sequences within genomic DNA of integrated viruses such as, but not limited to, HIV; yeasts; prokaryotes;

eukaryotic cells in culture; and cells in organs *ex vivo* or *in vivo*. Each of such methods could also be carried out with more than one oligonucleotide as described above.

An example of such a method utilizing HIV DNA would involve the introduction of DRAP protein or an episomal construct containing DRAP cDNA with modified HIV virus oligonucleotides or cDNAs. The exogenous or expressed DRAP would cut the genome-integrated HIV virus as directed by the mutated HIV oligonucleotides or cDNAs at the site of genomic integration, yielding an inactive HIV virus. This method could be used to inactivate any integrated gene or genes with known sequence.

Included within the scope of this invention is a mammalian cell or organ in which two or more genes have been knocked out. Such mammalian cells or organs are generated by repeating the procedures set forth herein for the generation of single knockouts, by introducing an additional oligonucleotide construct, or by breeding mammals, each with a single gene knocked out, to each other, and screening for those with the a double knockout genotype.

RNA Interference

The DRAP associated homology-dependent strand pairing and strand cleavage activities may promote heritable or non-heritable (transient) changes in gene expression by effectively attenuating, knocking out and/or knocking down levels of mRNA expressed from targeted genes through the phenomenon of RNA interference. RNA interference (RNAi) has been referred to in the literature (*See* Bosher, J.M. *et al*, Nat Cell Biol, 2000, 2(2): E31-36; and Hunter, C.P., Curr Biol, 1999, 17; 9(12): R440-2) and is also known as gene silencing. RNAi has applications in animal, plant and yeast cells. It is known to involve various genes and gene products but the complete set of genes and gene products involved is unknown. DRAP has the requisite activites to promote such an activity alone and/or in conjunction with endogenous cellular proteins.

Therapeutic Applications:

The ability to direct DRAP and oligonucleotide probes to specific cell-types

and target specific genes for disruption suggests certain therapeutic uses for this methodology. Certain pathologic conditions are known to result from the expression of mutant endogenous genes (p53, BRCA1, estrogen receptor "ER", et al.) or from expression of integrated viral genes (HIV-1, HIV-2, et al.). Similarly, upregulation of normal genes (e.g., drug transporters) may limit the effectiveness of conventional therapies. When DRAP and targeting probes are introduced by any of the methods described herein into affected cells, then the expression of the disease-causing or disease-promoting genes can be disrupted/ablated. This disruption/ablation can lead to the disorder being cured, ameliorated, or rendered susceptible to conventional therapies.

The use of DRAP and single-stranded (or double stranded with single stranded ends) probes to direct cleavages within endogenous genes that are then repaired by the cell, suggests the use of the methods described herein to be used in genetic therapy. A duplex DNA fragment, with a normal (wild-type) or mutant sequence embedded in the middle, that is flanked by specific single-stranded tails is directed to a specific locus within the genome containing sequences homologous to the single-stranded flanking sequence. DRAP-directed cleavages at each end of the fragment would permit the cell to repair the damage to the endogenous locus by replacement of the endogenous segment with the exogenous fragment. In this manner a simple cassette exchange is effected and the genotype of the cell modified.

Examples

EXAMPLE 1

Isolation and Initial Characterization of the *D. melanogaster* Recombination Associated Protein (DRAP) Gene

Drosophila embryonic nuclear extracts were fractionated by ammonium sulfate precipitation followed by column chromatography on S-Sepharose, SS-DNA agarose and gel filtration to isolate protein fractions with potent strand transferase activity. Figure 3A provides a schematic representation of the purification method used. Figure 3B shows the purification of fractions by elution from a single stranded (SS) DNA agarose column. The fractions were eluted from the SS DNA agarose column in a buffer

containing 500 mM NaCl. Protein fractions were serially concentrated on Centricon® centrifugal devices with nominal MW cutoff (NMWCO) filters of 100, 30 and 10 KDa. The material retained (R in Figures 3B and 3C) and filtered (F in Figures 3B and 3C) by each device was assayed for strand transferase activity with M13mpl8 linearized double stranded DNA (DS) and M13mp18 single stranded circular DNA (SSC) (Figure 3C). Figure 3C demonstrates that activity was present in the tested fractions because high molecular weight protein-DNA aggregates (AG) formed in addition to joint molecules and nicked circles. In the high salt fraction (500 mM NaCl) the active material behaved as if it were > 30 KDa. The high salt elution provides for the formation of multimeric DRAP complexes.

Figure 3D provides a silver-stained protein gel of the starting nuclear extract (NE) and active fractions eluted from the Sepharose (S), SS DNA (D) and Sephacryl HR-200 gel filtration (GF) columns. The activity co-enriched with a 20 KDa species at physiological salt (1 x PBS) during gel filtration.

Generating Antibodies to DRAP and Use of Anti-DRAP Antibodies

Highly purified fractions which retained DRAP specific activity, namely the ability to form joint molecules and high molecular weight protein-DNA aggregates, were used to generate polyclonal antisera in rabbits and monoclonal antibodies (IgM) by an *in vitro* method. (Reading, C.L., 1986, Methods Enzymol. 121: 18-27.) The IgM monoclonal antibodies were isolated by gel filtration from cell culture supernatants and selected for their ability to inhibit strand transfer activity. These antibody preparations proved useful for several purposes.

The IgM monoclonal antibody preparations, while more difficult to use due to their lower affinity as compared to IgG preparations, were used to identify a putative recombinase protein band on Western blots (Figures 3E and 3F) and to identify immunoreactive recombinase expression clones as summarized below. IgM antibodies generally have lower affinity than IgG antibodies but were of benefit here because of their monoclonal nature and because their lower affinity allowed for immunopurification of protein under native conditions.

First, the antibodies were used to identify active component(s) on Western blots (Figure 3E). Figure 3E shows a Western blot of from the Sephacryl HR-200 gel filtration column purification extracts reacted with both a rabbit polyclonal antibody (PAb) and a neutralizing mouse monoclonal IgM (Mab). The data show that a 20 KDa species was immunoreactive with the antibodies.

Second, the antibodies were used in the direct immunoaffinity purification of the active component(s) from Drosophila nuclear extracts (Figure 3F). Figure 3F depicts a Western blot demonstrating that a 20 KDa species was isolated directly from nuclear extracts on an IgM immunoaffinity column with small amounts of some other cross-reactive and/or interacting material.

Third, the antibodies were used to screen a Drosophila cDNA expression library in order to identify clones expressing immunoreactive peptide.

Fourth, the antibodies were used to look for immuno-crossreactivity with a number of known recombination-related proteins.

Isolation of Expression Clones and cDNA

Clones that were immunoreactive with two independent antibody preparations were plaque purified. The clone inserts were amplified by PCR and used for Southern blotting with a test oligonucleotide constructed from an amino acid sequence obtained from the presumptive immunoreactive recombinase protein band on a PVDF membrane.

One strongly immunoreactive clone was sequenced and the encoded protein denoted as the Drosophila recombination-associated protein (DRAP). The cDNA clone is 1378 bp in length [SEQ ID NO: 1] and the longest ORF is identified by [SEQ ID NO: 3] and codes for 169 amino acids [SEQ ID NO: 5], although the probable start codon is the Met at codon 11. The DRAP amino acid sequence defined by codons 11-169 is identified as [SEQ ID NO: 4] and is encoded by the DNA nucleotide sequence identified as [SEQ ID NO: 2].

EXAMPLE 2

DRAP Homology to Other Proteins

There are no significant homologues of the isolated DRAP cDNA or coding sequence as determined through BLAST data base searches. Nevertheless, using the full length Drosophila cDNA as a probe, there is cross hybridization on genomic Southern blots with human DNA. Furthermore, although the BLAST searches found no close amino acid sequence homology and thus no close protein homologues, the reaction with the anti-DRAP antibodies indicated that there was a weak relationship to *E. coli* RecA and a somewhat stronger relationship to both T4 Gene32 protein (a helicase) and (Bovine) Topoisomerase I.

The amino acid sequences predicted to be the most hydrophilic portions of the DRAP protein were identified. These presumptive antigenic sites were used to identify similar amino acid sequences in the immunologically cross-reactive proteins and to search the data bases. In this fashion a 10 amino acid motif [SEQ ID NO:15] was identified that is most conserved in the Flp site-specific recombinase (Figure 4D).

EXAMPLE 3

Localization of the DRAP Gene and Transcript

Polytene chromosome *in situ* hybridization studies using a digoxigenin labeled DRAP cDNA as a probe showed that the gene for this protein mapped to Drosophila chromosome band 63 C/D. This locus is where the recombination-defective mutation (meiS282) maps genetically.

Figure 5A depicts *in situ* hybridization studies on nurse cells in Drosophila embryos using a digoxigenin-containing antisense DNA single stranded probe prepared from the DRAP cDNA clone. Detection of the probe (purple staining) indicated that a maternally-encoded DRAP gene is transcribed in nurse cells of the ovary.

Figure 5B depicts *in situ* hybridization studies on Drosophila embryos using the same digoxigenin-containing antisense DNA single stranded DRAP probe. The data indicate that, following maternal expression of DRAP, the transcripts subsequently become uniformly distributed in the embryo. This suggests that the gene product may be involved in mitotic recombination, DNA repair processes and/or germ line development.

EXAMPLE 4

DRAP Expression

Recombinant forms of the DRAP protein were expressed *in vivo* from either the Met at the 11th or 60th codon in the ORF of the cDNA with either an N-terminal or a C-terminal (His6) purification epitope. The recombinant proteins expressed in bacteria were purified and examined for DNA strand transferase activity. The recombinant Drosophila recombination-associated protein (DRAP) was expressed from a modifed pRSET(B) vector in E coli strain HSM174(DE3) and contains 11 additional amino acids (incorporating the (His6) purification epitope) at the N-terminus of the open reading frame of the cDNA clone starting at the first Met (codon 11). Expression yielded a protein of approximately 21 kDa.

Recombinant proteins were poorly expressed in and difficult to purify from the insect cell/baculovirus system.

EXAMPLE 5

Protein Purification

DRAP

A schematic depiction of recombinant protein purification is provided in Figure 6A. Protein expression in *E. coli* was induced in a 500 ml Terrific Broth "TB" [10 grams bacto-tryptone, 5 grams bacto-yeast extract and 10 grams NaCl per liter, adjusted to pH 7.0] culture grown to OD600 - 0.7 by IPTG addition to 1 mM for 3 hours. The pelleted bacteria were frozen at -800C. Subsequently the pellet was resuspended in Basic Buffer "BB" [300 mM NaCl, 50 mM NaPi, pH=8.0] with 0.1% Tween 20 and lysed in a French Press. Insoluble material in the lysate was pelleted by centrifugation. The supernatant was filtered (0.2 uM) and passed over a 5 ml Ni-NTA column (Qiagen). The column was washed in 10 column volumes, each, of BB buffer with 0, 5 and 10 mM imidazole and the recombinant protein eluted with 3 column volumes of BB + 250 mM imidazole. The eluted material was concentrated (Millipore Ultrafree 10KDa device) desalted (Bio Rad 1ODG column) into 50 mM NaCl-containing column buffer [x mM

NaCl, 15 mM TrisHCl, 3 mM Mg Acetate, 1 mM EDTA, 0.4 mM PMSF, 10% glycerol, PH= 7.5] and put over a 5 ml phosphocellulose column. Weakly adherent proteins were washed away with up to 500 mM NaCl-containing column buffer and the recombinant protein was eluted with 1 M NaCl-containing column buffer. Fractions containing the recombinant protein were pooled, concentrated as before and loaded onto a 250 ml Sehpadex G 75 gel filtration column equilibrated with Transgenic Buffer ("TG") [10 mM NaCl, 10 mM Tris-HCl, 1 mM MgC12 and 0.1 mM EDTA, pH=7.5]. Individual fractions containing the recombinant protein were concentrated and examined by SDS-PAGE. (*See* Figure 6B.) Homogeneous fractions were pooled and filtered (0.2 uM). Protein concentration was determined by the Bradford method (Bio-Rad) with BSA as the standard. The final concentration of the pooled homogeneous material was 60ng/ul.

E. coli RecA and Single Stranded Binding Proteins

Homogeneous E. coli RecA protein (Promega) was diluted 1:1 with TG buffer and then desalted over several micro Bio-spin P6 columns (Bio Rad) equilibrated in TG buffer. The final concentration was 480 ng/ul and the material was diluted appropriately for use with TG buffer. Figure 6B shows both proteins and prestained MW markers (Benchmark, BRIL) after SDS-PAGE on a 12 % minigel stained with Coomassie Blue. SSB was similarly prepared in TG buffer.

EXAMPLE 6

Activity of DRAP

As described above, N-terminal, His6-tagged recombinant DRAP was purified successively over Ni-NTA (Qiagen), phosphocellulose (Whatman) and Sephadex 75 (Pharmacia) columns followed by centrifugal concentration (Millipore). This material was compared with commercial E. coli single strand binding protein (SSB, Promega) and RecA (Promega, Pharmacia and USB) in various assays.

The purified and commercial proteins (adjusted to 60 ng/ul) were seen on a Coomassie blue-stained 12% polyacrylamide gel: DRAP protein (5 and 2.5 ul) , SSB (5 ul), and RecA (5 ul; Pharmacia and USB). (Figure 6B.)

A. DRAP Produces Fewer Joint Molecules Than RecA/SSB

The strand transfer assay described in Example 1 and in Figure 3C above indicates that DRAP produced fewer joint molecules than RecA/SSB.

In a second standard strand transfer assay RecA/SSB (Promega) appeared to be more efficient than DRAP in producing joint molecules (JM) and nicked circles (NC) from double stranded linears (DSL) and single strand circles (SSC) than DRAP. (Figure 6C.)

The recombinant DRAP formed large protein-DNA aggregates (AG) with SSC and DSL substrates in a strand transferase assay in the presence of ATP (1 mM) and Mg (10M) but did not yield JMs or NCs after deproteinization with SDS. (Figure 6E.)

End labeling of the DSL used in these same strand transfer reactions indicated that DRAP did perform strand transfer in the ATP and Mg-containing reaction. (Figure 6F.) Before addition of SDS-containing loading dye, all the SS DNA and much of the DSL DNA were found in the protein-DNA aggregates (AG) at the top of the gel. Upon deproteinization, the label co-migrated with DSL and single stranded linear (SSL) species. Formation of the SSL product required both the SSC and DSL substrates and ATP. In addition, the SSL product was not formed from incubation of the DSL substrate alone with DRAP. (Figure 7.)

Standard Strand Transfer Activity Assay

	DRAP	RecA/SSB
DRAP (60 ng/ul)	1 ul	
RecA (240 ng)		1 ul
SSB (200 ng)		1 ul
ss DNA + ds DNA (50ng/ul, each)	2 ul	2 ul
10 X Tris (200 mM, pH=7.35)	1 ul	1 ul
10 X MgC12 (50-100 m M)	1 ul	1 ul
10 X ATP or H20 (10 mM)	1 ul	1 ul
ddH_2O	4 ul	3 ul
	10 ul	10 ul
Incubate at 37°C for 30 Min		
Loading solution (+SDS)	3 ul	3 ul
Total ul	13 ul	13 ul

Separate on a 0.8% 1 x TAE agarose gel at 4-6 V/cm for 1-2 hours with ethidium bromide in the running buffer (1 x TAE) or post stained and followed by photo-documentation.

Loading solution: 1 x TAE, 50% glycerol, 2.5% sodium docecylsulfate ("SDS'") and bromophenol blue ("BPB").

The standard strand transfer assay experiments described above indicate that DRAP does not stop with the generation of intermediate products (JM or NC) as is observed with RecA/SSB (or RAD 51/RAD 52). The reaction with DRAP alone progresses to complete resolution of the recombination products similar to those observed when utilizing a reconstituted recombination system containing RecA/SSB with resolving endonucleases Ruv A, B and C. Thus, DRAP provides for the ability to insert or modify small pieces of single stranded or double stranded DNA directly within genes using "oligonucleotides", *i.e.*, less than about 100bp into the gene of interest.

B. DRAP Possesses Topoisomerase Activity

Various recombinases exhibit topoisomerase (1) - like activity. When the protein is incubated with a supercoiled plasmid one strand of the DNA is cleaved and may be religated. If the other DNA strand passes through a transient nick then the plasmid is relaxed. Nicked DNA may even be linearized. This activity is exhibited by DRAP and to a lesser extent by RecA (+ single strand binding protein, SSB). This activity is Mg - dependent but independent of ATP.

In a topoisomerase assay (see assay conditions described below), an M13mp18 plasmid preparation containing both supercoiled (SC) and nicked circular (NC) DNA forms was incubated with DRAP (60 ng) or RecA/SSB (240/200 ng) at 37°C for 30 min. The reaction was stopped with SDS-containing loading dye and the products were separated by agarose gel electrophoresis. DRAP produced double strand linear (DSL) DNA molecules in an ATP-independent reaction to a significantly greater degree than RecA/SSB. (Figure 6D.)

Topoisomerase Activity Assay

	DRAP	RecA/SSB
DRAP (60 ng/ul)	1 ul	
RecA (240 ng)		1 ul
SSB (200 ng)		1 ul
Plasmid (50 ng / ul)	1 ul	1 ul
10 X Tris (200 mM, pH=7.35)	1 ul	1 ul
10 X MgC12 (50-100 m M)	1 ul	1 ul
10 X ATP or H20 (10 mM)	1 ul	1 ul
ddH_20	5 ul	4 ul
	10 ul	10 ul
Incubate at 37°C for 30 Min		
Loading solution (+SDS)	3 ul	3 ul
Total ul	13 ul	13 ul

Separate on a 0.8% 1 x TAE agarose gel at 4-6 V/cm for 1-2 hours with ethidium bromide in the running buffer (1 x TAE) or post stained and followed by photo-documentation.

Loading solution, 1 x TAE, 50% glycerol, 2.5% SDS, BPB

The data shown in Figure 6D demonstrate that the presence of DRAP topoisomerase activity produced a conversion of SC to NC and NC to linear forms of DNA with the appearance of a new linear band.

C. DRAP Does Not Possess ATP-Dependent Helicase Activity

Figure 7 shows that DRAP does not possess ATP-dependent helicase activity. The Figure shows that in a standard three strand reaction using DRAP as described in Section A above, a displaced single stranded DNA band is produced. The displacement of one strand from a labeled duplex DNA is not the consequence of an ATP-dependent helicase reaction. The data show that no strand displacement occured due to unwinding or helicase activity from duplex DNA incubated with DRAP, either alone or in the absence or presence of ATP. Very limited DNA displacement occured when incubated with a homologous SSC, but no strand displacement occurred when incubated with

homologous supercoiled plasmid double stranded DNA ("SCP") in the absence of ATP. Significant strand displacement from the labeled DSL DNA occurred only when a homologous SSC, but not SCP, was present and co-incubated with DRAP and ATP.

The data demonstrate that incubating DRAP with either double stranded DNA (2 strands) or double stranded and supercoiled plasmid DNA (4 strands) produces no product, while incubating double stranded and single stranded circular DNA (3 strands) provides for homologous recombination. Thus, all of the data described above demonstrate that DRAP exhibits a homology- and ATP-dependent three strand transfer activity coupled with a topoisomerase I-like ATP independent cleavage activity.

D. DRAP Does Not Possess Nuclease Activity

An assay was performed to determine if any nuclease activity, that would degrade the mutagenic oligonucleotides to be used, is present in the recombinant DRAP samples. The assay conditions are outlined below:

Nuclease Activity Assay

Protein Sample	(60 ng/ul)	1 ul
Oligonucleotide	(5 ng/ul)	1 ul
10 X Tris	(200 mM, pH=7.35)	1 ul
10 X $MgC1_2$	(100 MM)	1 ul
10 X ATP(γ-S)	(10 MM)	1 or 0 ul
ddH_20		5 or 6 ul
		10 ul
Incubate at 37°C for 30 Min		
Loading solution (+SDS)		3 ul
Total ul		13 ul

Separate on an 8x10cm 6% 1 x TAE minigel (MiniProtean, BioRad) at 250 V for 14 minutes. Dry gel onto filter paper and expose to film.

Loading Solution- 1 x TAE, 50% glycerol, BPB and xylene cyanol.

Any oligonucleotide may be used for this assay. For this experiment, an

oligonucleotide (33-mer) derived from the central portion of the pUC19 poly linker 5'-GGTACCCGGGGATCCTCTAGAGTCGACCTGCAG-3' [SEQ ID NO:20] was endlabeled at the 5' end with ^{32}P-ATP using T4 polynucleotide kinase. The oligonucleotide was purified on Qiaquick columns using buffer PN (Qiagen) and the OD at 260 nm indicated a concentration of 5 ng/ul. Any oligonucleotide sequence may be used in this experiment. The SEQ ID NO:16 oligonucleotide was incubated with 60 ng of DRAP or RecA in various buffers for 30 minutes at 370C. A loading buffer was added and the material separated on a 6% 1 x TAE, polyacrylamide minigel at 250 V for 14 minutes. The gel was dried and exposed to film with one intensifying screen for ~16 hrs. at - 800C. No degradation of the oligonucleotide was observed. (Figure 8.) Gel shifting with RecA in the presence of ATP-γ-5 is consistent with the known properties of that protein. The data show that DRAP does not posses nuclease activity.

The data described above enable postulation of a non-limiting model of the mechanism of action of DRAP. The model is depicted in Figure 9 and is not meant to limit the invention to this mechanism. The schematic depicted in Figure 9 indicates how DRAP may act to couple its strand transfer and topoisomerase activities. In an ATP-independent reaction DRAP can relax and linearize a supercoiled (SC) plasmid substrate. DRAP can also form joint molecules (JM) and nicked circles (NC) from double stranded linear (DSL) and single stranded circular (SSC) substrates as is similar to RecA/SSB activity. While the reaction stops at the JM/NC stage for RecA/SSB, it progresses further for DRAP due to its topoisomerase activity. With recombinant DRAP the JMs progress completely to NCs and the NCs are converted back to DSLs. The position(s) of the cleavage(s) have not been determined and the possible putative products are indicated in Figure 9.

EXAMPLE 7

Initial Generation of Transgenic Mice

Initial transgenic experiments were carried out using DRAP and mutant oligonucleotides (mutant nucleotide is underlined and bold) corresponding to the N-myc, 5'-TTTCCTGAAAAGC**T**TATTCAGCACCCGAA-3' (Sense Strand) [SEQ ID NO: 21]; β-1 Globin, 5'-ATGGTGCACCTGACTGATG**T**TGAGAAG-3' (Sense Strand) [SEQ ID NO: 22]; and Agouti genes. (*See* Figure 11.)

The amount of olignucleotide DNA for the injections was based upon estimates of the number of "gene-sized" DNA molecules injected in a routine transgenic experiment. It was estimated that approximately 500 molecules per picoliter ("pl") were injected in a typical transgenic experiment. This translates to a concentration of approximately 7.5 ng/ml for a 30-mer oligomer. In a preferred embodiment, the mutagenic oligonucleotide used should be hydroxylated at the 5' end ("5'OH").

Three different ratios of protein per nucleotide were examined for each of the co-injections. The "Low" ratio was equal to 1 protein molecule for each oligonucleotide. The "Medium" ratio was consistent with the observed ratio for RecA of 1 protein molecule for every three nucleotides (10 protein molucules/ 30-mer). The "High" ratio was one factor of ten above the observed value for RecA which gave a final ratio of 100 protein molecules per 30-mer. There was no observed toxicity to the embryos at any of the co-injection ratios.

As seen in Figure 11, phenotypic changes were observed with the "High" ratio. The still-born phenotype for N-myc targeting is consistent with an embryonic lethal, *i.e.*, a conventional "knockout". The runt phenotype for βl-globin targeting is consistent with a potenial change in oxygen carrying capacity of β or highly homologous globins in mice during embryonic and/or fetal development. Thus this ratio of protein to oligonucleotide was the starting point for all further transgenic studies. The toxicity to the embryo of increasing the amount of DNA and/or protein should also be determined prior to reimplantation of injected zygotes. The data for these experiments is provided in Figure 11. That data show that experiments utilizing 5'OH-containing oligomers directed to the N-myc Exon1 and β1-globin genes produced phenotypically transgenic mice, while mice

generated with the Agouti 5'OH oligomer produced no obvious phenotypic change.

It was determined through restriction analysis of DNA from multiple animals that a direct replacement of the N-myc and βl-globin loci did not occur.

EXAMPLE 8

Additional Transgenic Experiment

Selection of Additional Target Gene

The mouse genetic database was examined for candidate genes in order to test the ability of DRAP to produce targeted mutations in transgenic mice. The criteria for inclusion were:

1. The method should be able to reproduce a known point mutation.
2. The known mutation should be associated with an observable phenotype that is easily confirmed by molecular methods.
3. The mutation/phenotype should be autosomal dominant or semi-dominant.
4. The mutation/phenotype should not be lethal even when homozygous.

Using these criteria, the c-Kit tyrosine kinase receptor was selected. Mutations in this gene are responsible for the white spotting phenotype. The best allele for mutagenesis is W-42. This missense mutation produces a Restriction Fragment Length Polymorphism (RFLP) that is capable of being evaluated by cleavage of a PCR product. Furthermore, there are only a few related sequences in the data base with similarity to the proposed mutagenic oligonucleotide.

The portion of the gene that was determined to be suitable for modification along with the new RFLPs that would be generated are shown in Figure 12. Because coat color variation is readily apparent and can be demonstrated in a photographic record, we attempted to modify the c-kit gene with a mutagenic oligonucleotide.

Injection of DRAP and c-kit Oligos

DRAP was coinjected into mouse embryonic stem cells with c-kit 5'P-containing oligonucleotide, [SEQ ID NO:9] at a concentration of 10:1 molar ration of

DRAP to oligonucleotide. In this experiment approximately 500,000 molecules of DRAP and approximately 50,000 molecules of oligonucleotide per embryo were injected using the standard transgenic protocol as described in U.S. Patent No. 4,873,191.

Analysis of the transgenic animals for the mutant c-kit are carried out at two levels.

The first level of analysis is to assess for visual evidence of the white spotting phenotype. Figure 13 depicts a photograph of a white spotted mouse produced from the co-injection of DRAP and the mutagenic c-kit oligonucleotide as well as an unspotted control mouse. The data depict a disruption of the wild type gene sufficient to alter the gene product of the embryo, ablate gene function and produce the expected white-spotted phenotype.

The second level of analysis is to perform molecular analysis of the c-kit gene for evidence of a gene conversion event. The molecular analysis is performed by genomic DNA PCR amplification of either the complete or a partial fragment from exon 17 utilizing the PCR probes identified as [SEQ ID NO:9 and 10]. This is followed by determining if the new Apo I or Tsp 509 1 restriction sites are created in the gene conversion event. Because the exon size is small (124 bp) and the cleavage is near one end of the fragment, the restriction products are to be separated on a 6% polyacrylamide gel.

For restriction analysis Tsp5091 is, perhaps, the better choice of enzyme to use because Apo I is subject to "star" activity. Star activity could lead to false positive results. For final confirmation, the PCR material can be cloned into a standard vector and then sequenced. All of the critical enzymes needed are commercially available (NEB and others).

All of the references identified hereinabove, are hereby expressly incorporated herein by reference to the extent that they describe, set forth, provide a basis for or enable compositions and/or methods which may be important to the practice of one or more embodiments of the present inventions.

1. (Amended) An isolated nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

2. (Amended) An isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide of SEQ ID NO: 4.

3. (Amended) An isolated nucleic acid that hybridizes to a nucleic acid as defined in claim 1 under high stringency hybridization conditions.

4. A nucleic acid vector comprising a nucleic acid as defined in claims 1 or 2 operably linked to a transcription regulatory element.

5. A cell comprising a vector as defined in claim 4.

6. A cell as defined in claim 5, wherein said cell is selected from a group consisting of bacterial, fungal, insect, and mammalian cells.

7. A method for producing a polypeptide, which comprises:

(i) culturing a cell as defined in claim 5 under conditions suitable for the expression of DRAP polypeptide; and

(ii) recovering said polypeptide from said culture.

8. An isolated polypeptide comprising the amino acid sequence defined by SEQ ID NO: 4.

9. An antibody that specifically recognizes Drosophila Recombination Associated Proten (DRAP) polypeptide.

10. A fragment of a DRAP polypeptide of SEQ ID NO: 4 or function-conservative variants of said polypeptide, said fragment or function-conservative variant being characterized in that, it carries out recombinase/topoisomerse activity associated with the DRAP protein.

11. A method for isolating genomic DNA comprising introducing an oligonucleotide and DRAP into a cell, homologously recombining said oligonucleotide with genomic DNA homologous to said oligonucleotide and isolating said genomic DNA.

12. A method for targeting mutagenesis of a defined segment of DNA comprising introducing DRAP and an oligonucleotide homologous to said DNA segment into a cell.

13. A method for the removal of a defined segment of DNA comprising introducing DRAP and an oligonucleotide homologous to said DNA segment into a cell.

14. A method for cloning a defined segment of DNA comprising introducing DRAP and an oligonucleotide homologous to said DNA segment into a cell.

15. A method for mapping a defined segment of DNA comprising introducing DRAP and an oligonucleotide homologous to said DNA segment into a cell.

16. A method of promoting gene disruptions of a defined segment of DNA comprising introducing DRAP and an oligonucleotide homologous to said DNA segment into a cell.

17. A method for the experimental and therapeutic application of DRAP driven genetic modification of a gene responsible for a genetic disease comprising introducing DRAP and an oligonucleotide homologous to said gene into a cell.

18. A protein sample comprising an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4, said polypeptide comprising at least about 1% by weight of the total protein in the sample.

19. A protein sample comprising an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 4, said polypeptide comprising at least about 10% by weight of the total protein in the sample.

10031893 .071902

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: **ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY** [US/US]; 1300 Morris Park Avenue, Bronx, NY 10461 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **EISEN, Andrew** [US/US]; 12412 St. James Road, Rockland, MD 20850 (US).

(74) Agents: **ZITRON, Anne, E.** et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022-7513 (US).

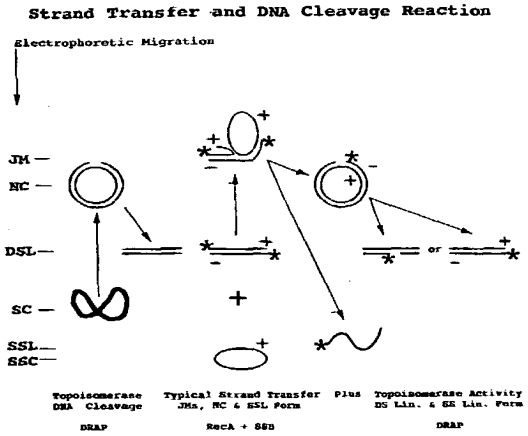
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DROSOPHILA RECOMBINATION-ASSOCIATED PROTEIN AND METHODS FOR USE



(57) Abstract: The invention encompasses Drosophila Recombination Associated Protein (DRAP) isolated from *D. melanogaster* and a nucleic acid sequence encoding DRAP. The Drosophila Recombination Associated Protein, its homologues from other organisms or active peptides derived therefrom, as well as DNA encoding such protein are useful for homology-dependent pairing of three DNA strands. The combination of strand-transfer and topoisomerase activities associated with DRAP permits directed pairing and cleavage at defined site(s) within DNA. This in turn makes possible the isolation and/or removal of a defined segment of DNA. DRAP is also useful in cloning, genomic cloning and gene mapping, in promoting gene disruptions or "knockout" mutations, in carrying out targeted mutagenesis of specific genes and in generating transgenic animals. The invention further encompasses a method for experimental and therapeutic application of DRAP driven knockouts or other modifications of genes responsible for genetic diseases as well as the use of DRAP driven genetic manipulation of genes in gene therapy.

WO 01/07627 A1

Fig. 1

DRAP Complete cDNA Clone Insert

```
agcgattacg gtagagatat ggtaatgcaa cggtggatgt gaactccttg      50
gtttgcggtg aatgcgttca acggtggatc ttcgcttgca cctttcgcga     100
tagacgacat acggatacag atacagatac agaatggcct ccaacaacag     150
tagtaccacc gatctggaca gccaggtcaa tgtggaggat ttgcccataa     200
cgttcaaggt gaagtacatt ggttccgaag tggcacgtgg cttatggggc     250
attaagtata cgcgtcgtcc ggttgacata atggtgggcg tggccaagaa     300
cctgccgccc aataaggtgc tgcccaactg cgaactgaag gtgtccaccg     350
acggagtcca gctggagatc atatcgccaa aggccagcat caatcactgg     400
agctatccca tcgacacgat ctcgtatggc gttcaggacc tggtctacac     450
aagggtcttt gccatgatcg tggtgaagga cgagtcgagt ccgcatccct     500
ttgaggttca cgccttcgtg tgcgacagtc gtgcgatggc gcggaagttg     550
acctttgccc tggccggccg ccttccagga ttactcgcga cgggtcaagg     600
aggcaaccgg tgaggaggag ggcgaggcca cgcccagcga cactattaca     650
cccacgcgac acaagttcgc catcgatctg cgaacgccgg agaaatccag     700
gctggcgaac tggagcagga aacggaggcg tagttatcct ggtgatcctg     750
cgttggctcc gtcaatgaga tgtgatgtgt tagttactta acgtccagtg     800
ttcactgtat ctgtaaattg tggttctctc acctggtagt tgcctcatac     850
agctaattac ccaaagccta agtgttaata cgatttgtaa acgatttcta     900
aaataaatta cgaatatggt atgtttggct atttgaattg ggctacaacc     950
tgttgatatg ccacttggca aaaaaaaaaa acgccagcac caattctttt    1000
acttctgttt cttgtgaccg acataaaaga tgcaccaaag ctgctattcc    1050
accagcgttc tttattccac gcttgttttc atcattttgt cttccgtaag    1100
ataaattacg taaagcacca caggcatttt tatgtatttc tggagaatca    1150
taagatagca gtcgaactaa tggtggtata cctcccagag atcttgtacg    1200
ttgcttgttt ggatcatcca tgtagcacaa atgctgtaga taggctgctg    1250
cattagcttt tatagcacta ctcgggttgc ttaaaaagct tattacttct    1300
gaaagatttg gatcccgcca tctcattgta gaacaaatat cattttctga    1350
tccttcaatg taatcatcct tttcttcc                           1378
```

Longest ORF	nt 104 - 610
Probable Start Codon	nt 134
CDS Expressed as Recombinant Protein	nt 134 - 610

Figure 2

DRAP Coding Sequence - Restriction Map

With 206 enzymes: * MaxCuts: 1

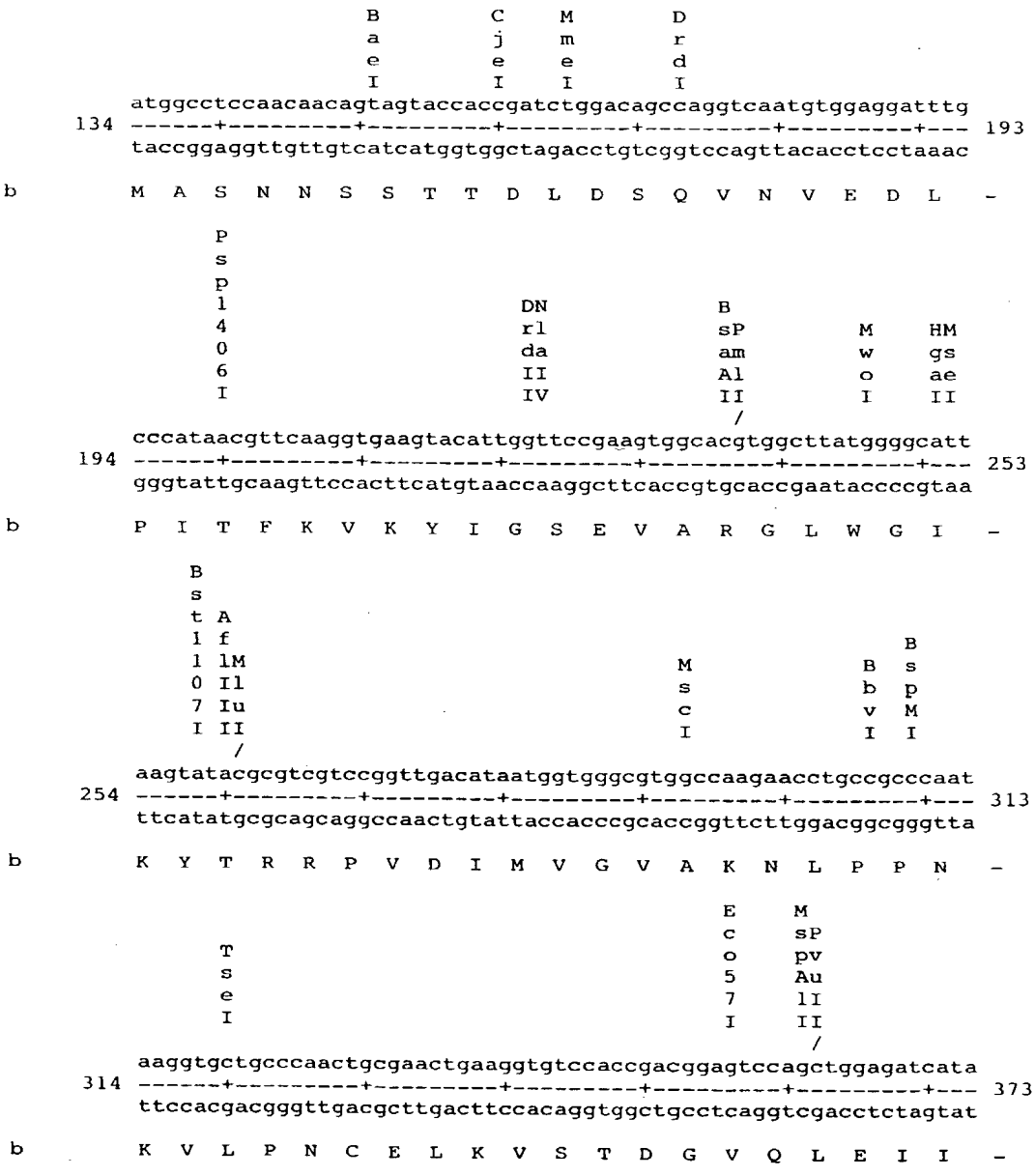
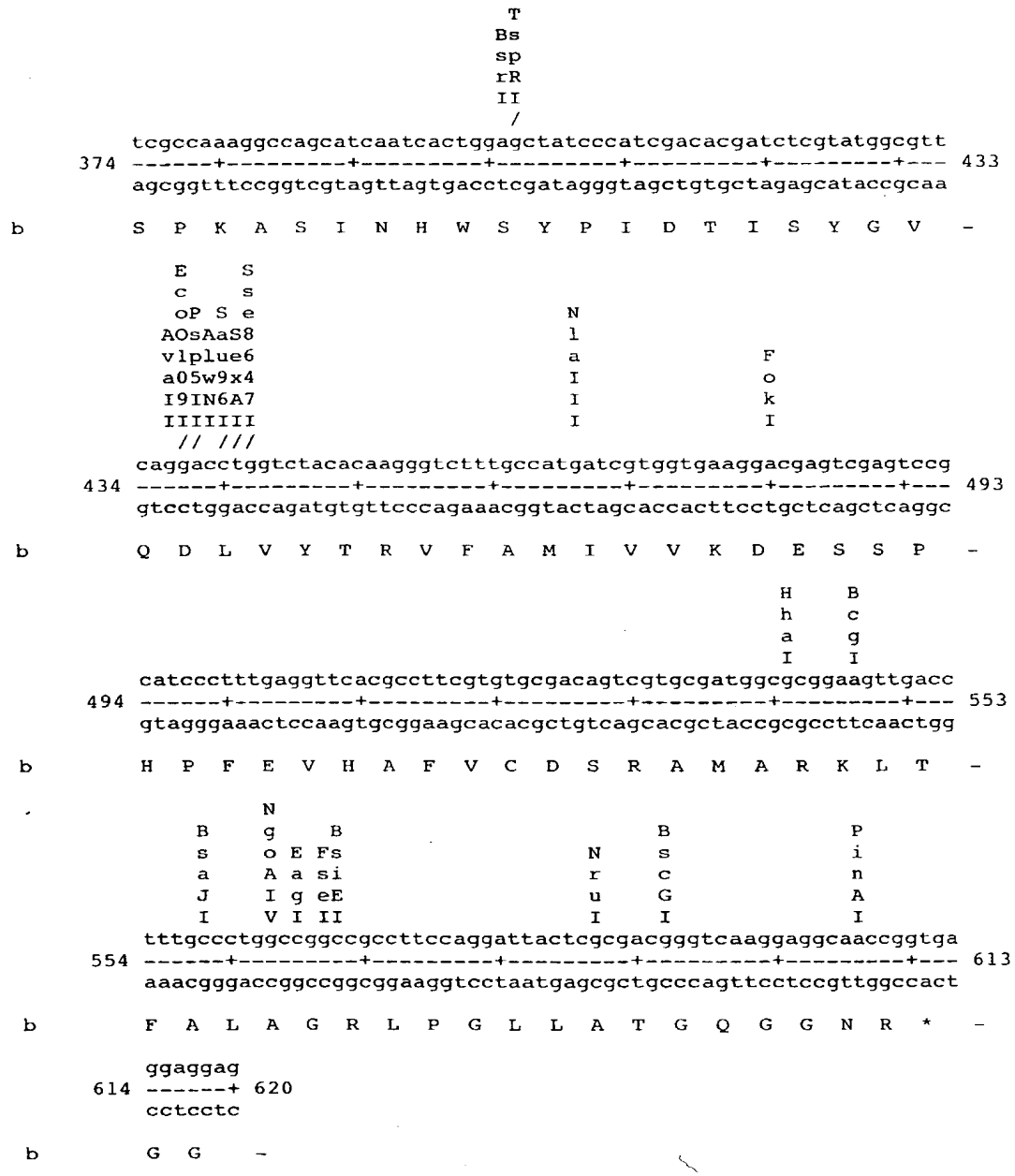
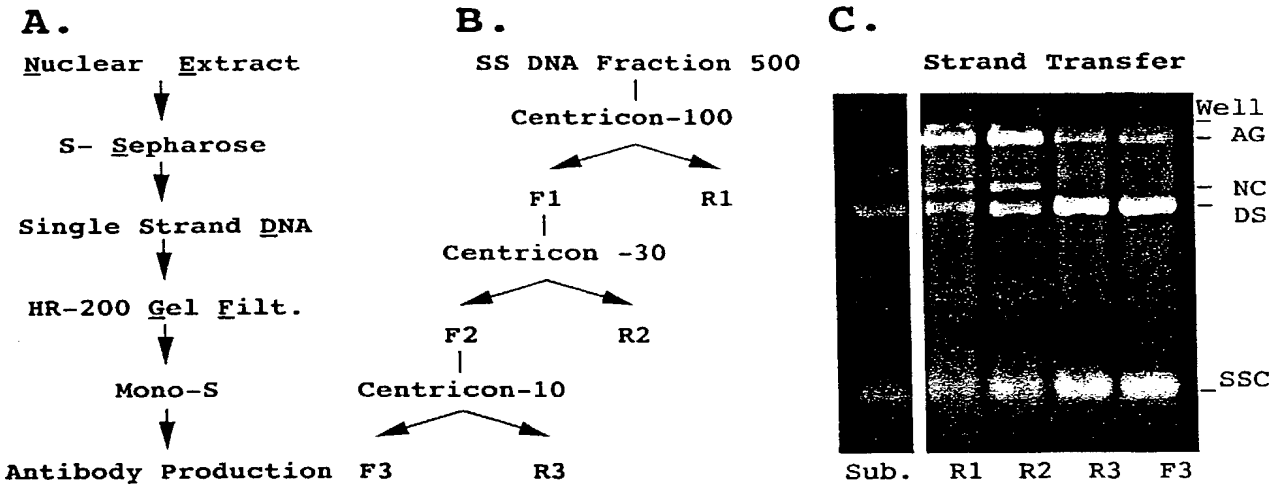


Figure 2 (continued)



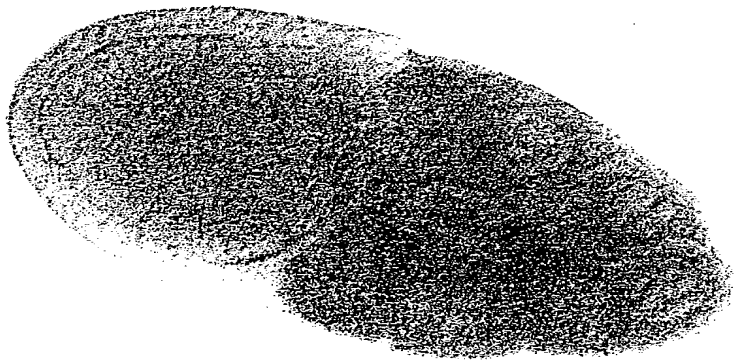
Figures 3A-3F

Recombination-Associated Protein Purification

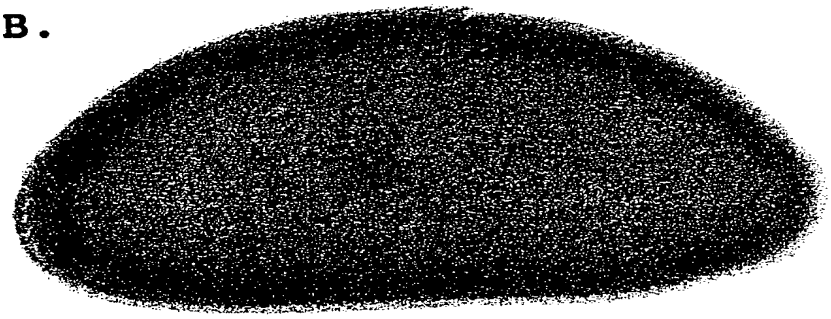


DRAP Transcript Distribution

A.



B.



Figures 5A and 5B

Figures 4 A-4D

Recombination-Associated Protein cDNA Clone

A.

DRAP: 1378 bp Eco RI insert
ORF 104-610

1	134	281	902	970	1378
	ATG	ATG	AATAAA	(A)12	(A)29
	#1	#50			



B.



```
MASNNSSTTD  LDSQVNVEDL  PITFKVKYIG  SEVARGLWGI  KYTRRPVDIM    50
                                                      2
VGVAKNLPPN  KVLPNCELKV  STDGVQLEII  SPKASINHWS  YIPDTISYGV   100
QDLVYTRVFA  MIVVKDESSP  HPFEVHAFVC  DSRAMARKLT  FALAGRLPGL   150
                  1                       3
LATGGGGNR                                                   159
```

C.

Potential	D1-------(30 to 70)---D2---(35±)---E1	Motifs
AA#s	(10 or 12)---(36 to 38)---48---(30)--- 78	
	(19)---(54)---73---(44)---117	
	(19 or 48)---(46 to 75)---94---(30)---124	

D.

Rad 51	Mouse - Human	L L I V - D - S
Rad 51	Yeast	L I V V - D - S
DCM 1	Yeast	L I V V - D - S
RecA	E. Coli	V I V V - D - S
Drosophila DRAP		M I V V K D E S S P
FLP Recombinase		M I A L K D E T N P
T4 Gene 32 Protein		I L V V K D P A A P
		M I A V - DVE M G E
		K - GFS S E
Human Topoisomerase I		I K D E - - P
		K D G S S E
		GFS S P

Figures 6A - 6F

Recombination-Associated Protein Activities

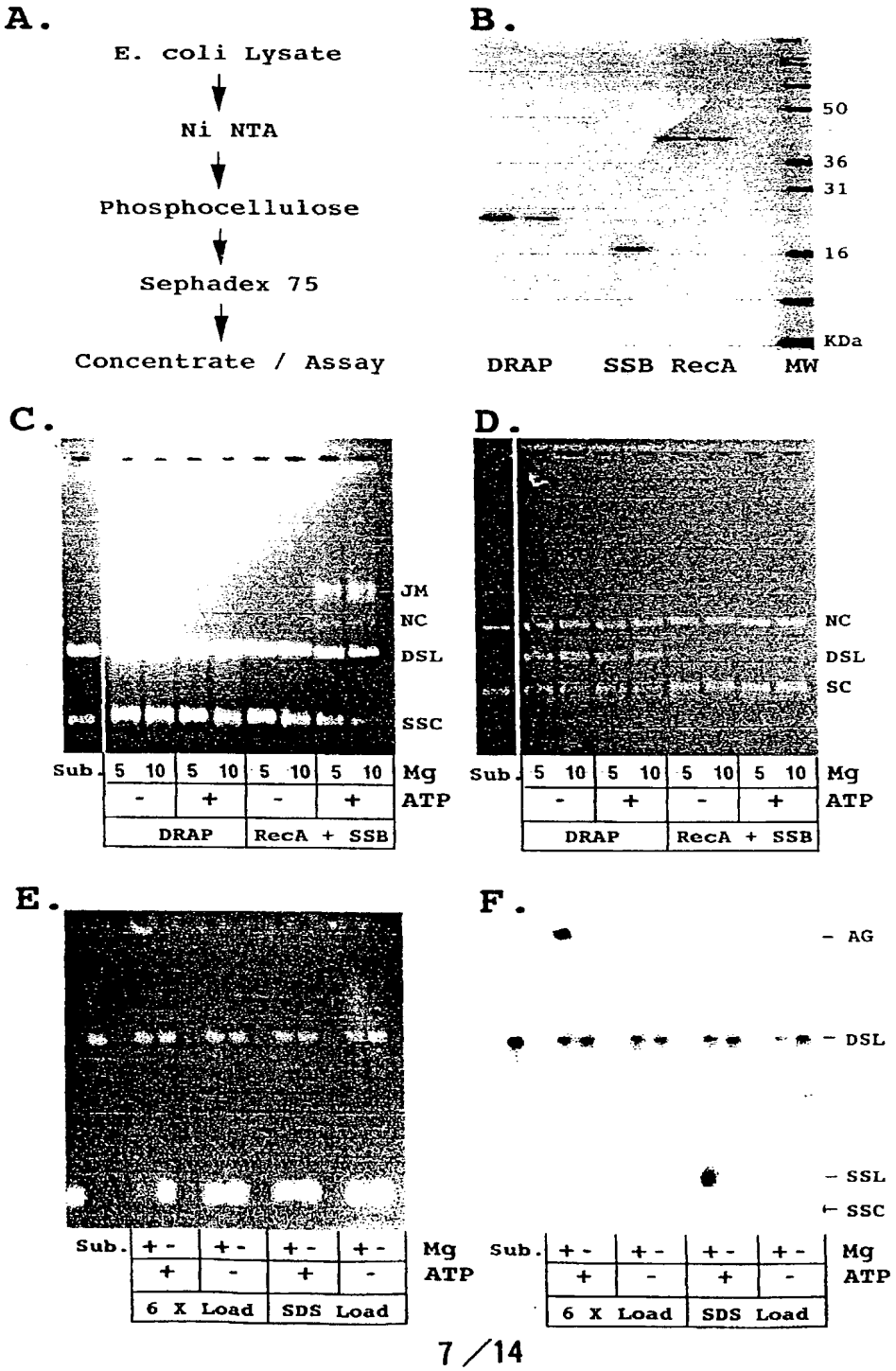


Figure 7

ATP-Dependent Three Strand Exchange Reaction

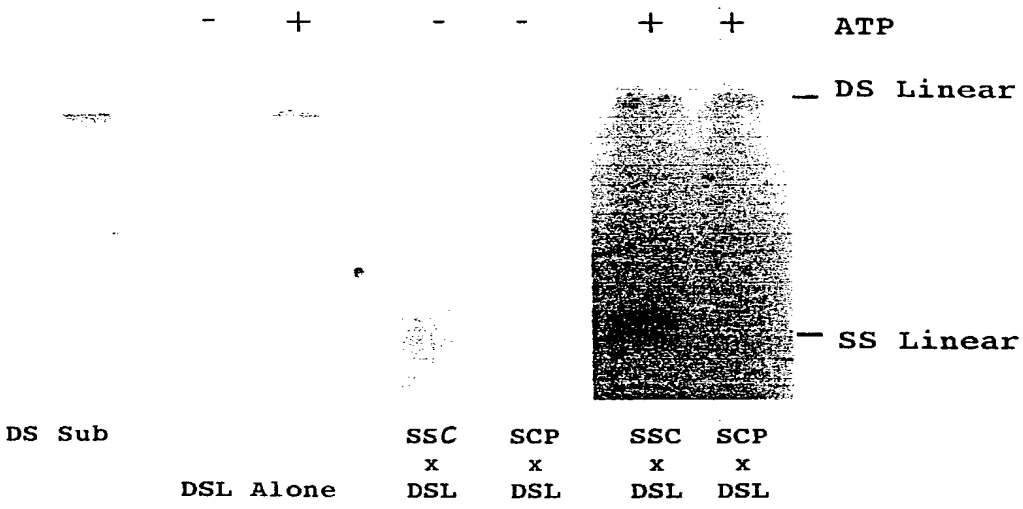


Figure 8

Nuclease Assay - 5' ^{32}P Oligo

(6% 1X TAE PAG)

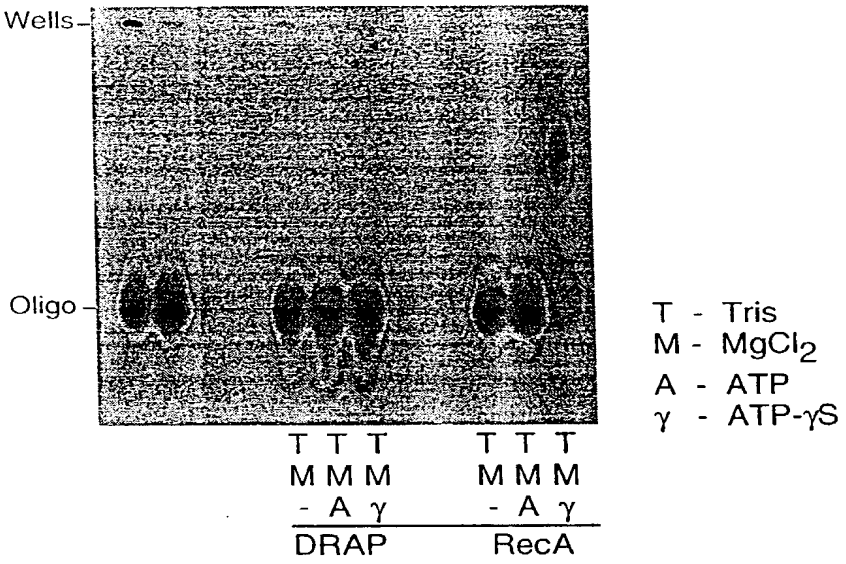
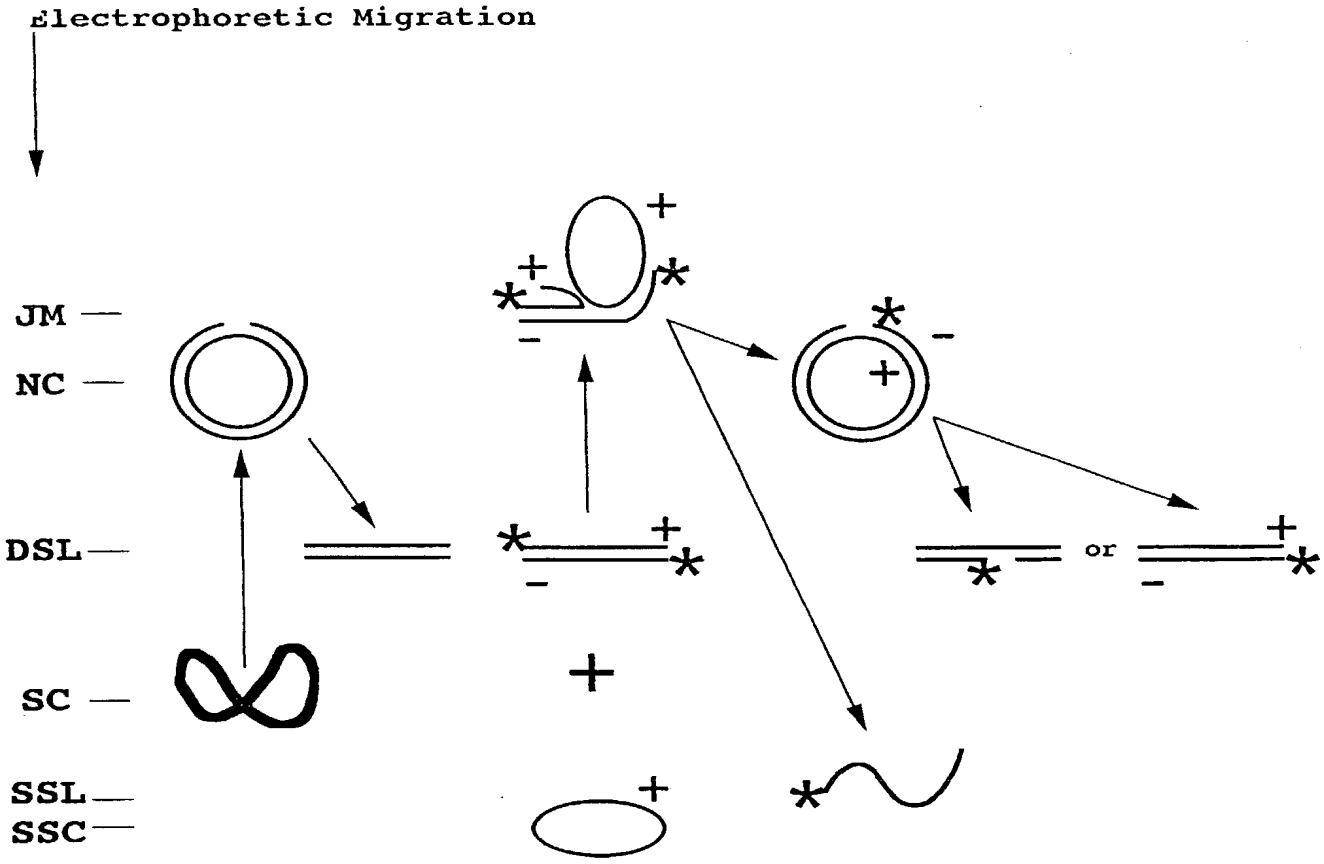


Figure 9

Strand Transfer and DNA Cleavage Reaction



Topoisomerase DNA Cleavage — DRAP

Typical Strand Transfer JMs, NC & SSL Form — RecA + SSB

Plus Topoisomerase Activity DS Lin. & SS Lin. Form — DRAP

Driving A Gene Conversion With A Recombinase and Oligos

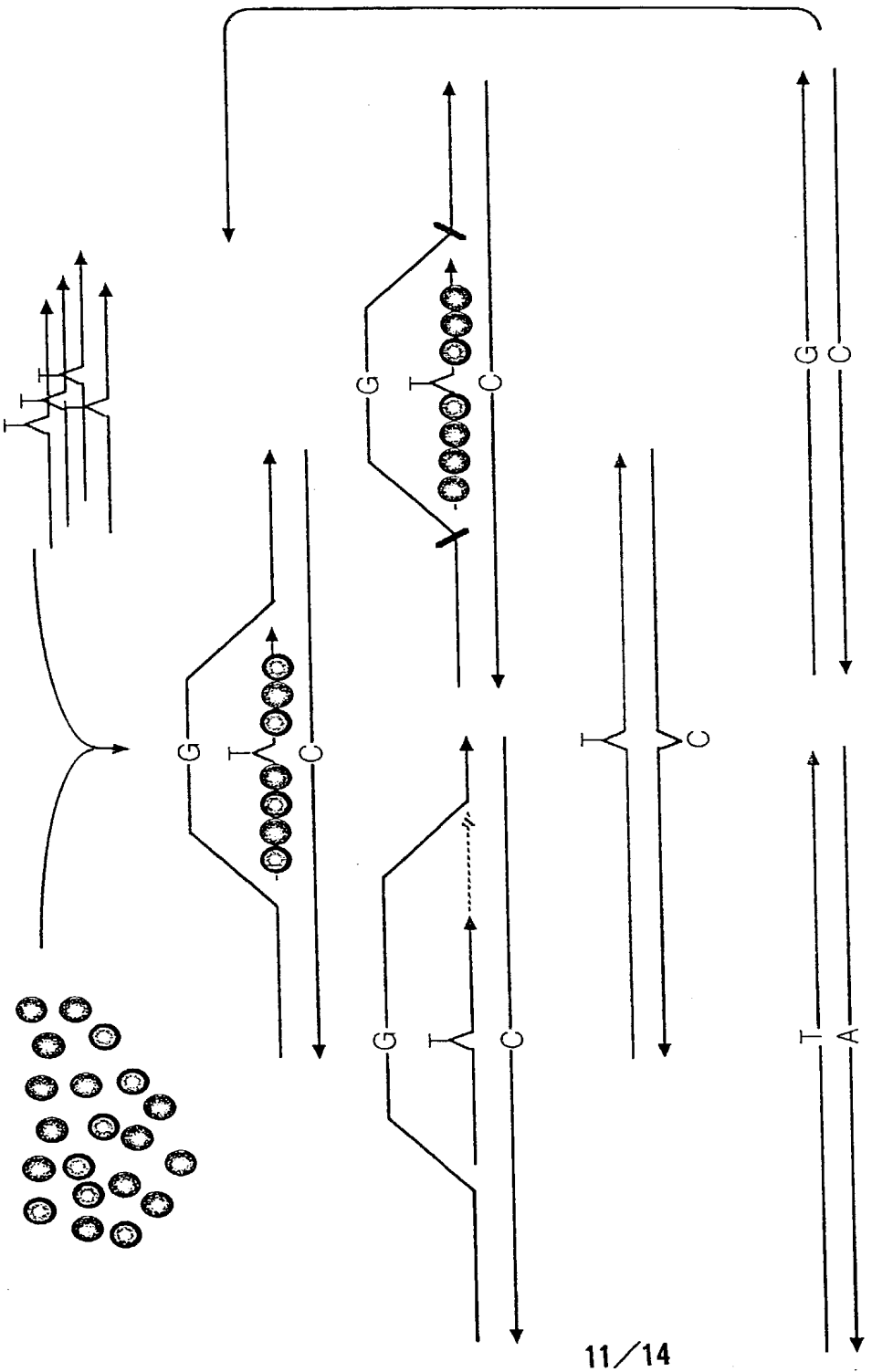


Figure 10

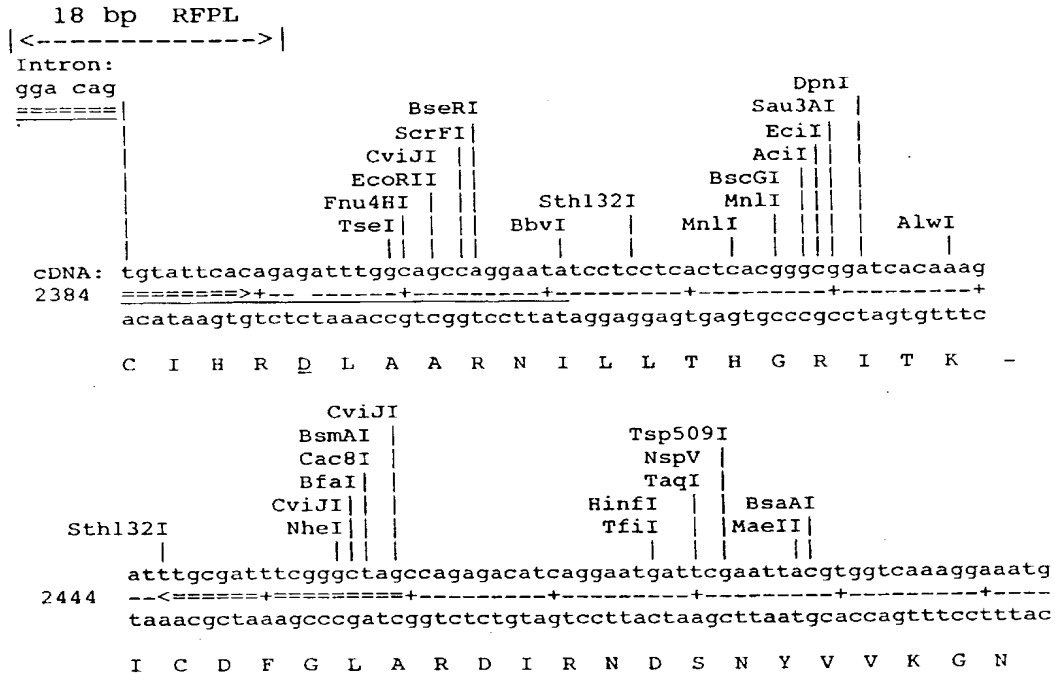
Figure 11

Targeted Transgenics

Gene	Oligo (No Phenotype)	Oligo + DRAP (Molar Ratio - Protein : Oligo)	
1. N - myc (Exon 1)	16	Low (1:1)	60 OK
		High (100:1)	4 Alive
			11 Stillborn
2. β1 globin (Ala --> Val)	7	High (100:1)	10 OK
			5 Runts
			4 OK
			1 Sickly
3. Agouti	11	High (100:1)	8 OK

Figure 12

Murine c-Kit Exon 17



W42 Mutation



Underline: Potential Mutagenic Oligo

=====> <====== Potential PCR Primers for RFLP Analysis

Figure 13

Targeted Transgenic Mice



White-Spotted Mutant

Control Mouse

10/031893
JC13 Rec'd PCT/PTO 22 JAN 2002

EXPRESS MAIL CERTIFICATE

Date 1/22/02 Label No. EL767722429US

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

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Customer No.: 07278

PATENT TRADEMARK OFFICE

Docket No: 2567/1F496-US2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Andrew EISEN

Serial No.: T.B.A.
National Phase Application of
PCT/US00/19901

Art Unit: T.B.A.

Filed: Concurrently herewith

Examiner: T.B.A.

For: **DROSOPHILA RECOMBINATION-ASSOCIATED PROTEIN AND METHODS FOR USE**

LETTER TO OFFICE DRAFTSMAN

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

January 22, 2002

Sir:

Subject to the approval of the Examiner, please amend Figure 4 of the subject application as follows: In the second line of Figure 4B, replace the sequence "IP" with the sequence "PI". In the last line of Figure 4B, replace the second occurrence of the letter "G" with a "Q". A clean copy of amended Figure 4 and a marked-up copy of Figure 4 showing the changes in red are attached.

Respectfully submitted,

M Scott Bernstein

Mitchell Bernstein, Ph.D.
Reg. No. 46,550
Agent for Applicants

DARBY & DARBY, P.C.
805 Third Avenue
New York, N.Y. 10022
Phone (212) 527-7700

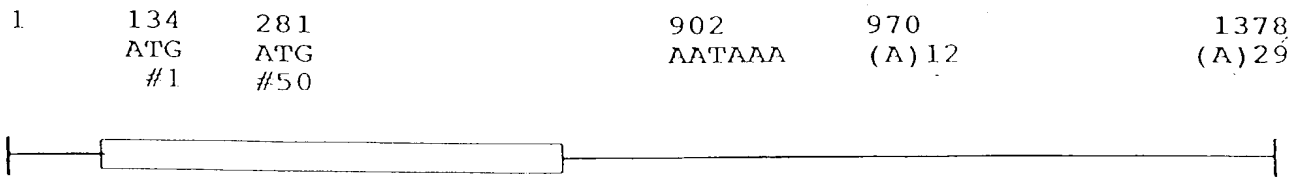
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Recombination-Associated Protein cDNA Clone

10/031893

A.

DRAP: 1378 bp Eco RI insert
ORF 104-610



B.

MASNNSSTTD	LDSQVNVEDL	PITFKVKYIG	SEVARGLWGI	KYTRRPVDIM	50
VGVAKNLPPN	KVLPNCELKV	STDGVQLEII	SPKASINHWS	YPIDTISYGV	100
QDLVYTRVFA	MIVVKDESSP	HPFEVHAFVC	DSRAMARKLT	FALAGRLPGL	150
LATGQGGNR					159

C.

Potential	D1-------(30 to 70)---D2---(35±)---E1	Motifs
AA#s	(10 or 12)---(36 to 38)---48---(30)--- 78	
	(19)---(54)---73---(44)---117	
	(19 or 48)---(46 to 75)---94---(30)---124	

D.

Rad 51	Mouse - Human	L L I V - D - S
Rad 51	Yeast	L I V V - D - S
DCM 1	Yeast	L I V V - D - S
RecA	E. Coli	V I V V - D - S
Drosophila DRAP		M I V V K D E S S P
FLP Recombinase		M I A L K D E T N P
T4 Gene 32 Protein		I L V V K D P A A P
		M I A V - DVE M G E
		K - GFS S E
Human Topoisomerase I		I K D E - - P
		K D G S S E
		GFS S P

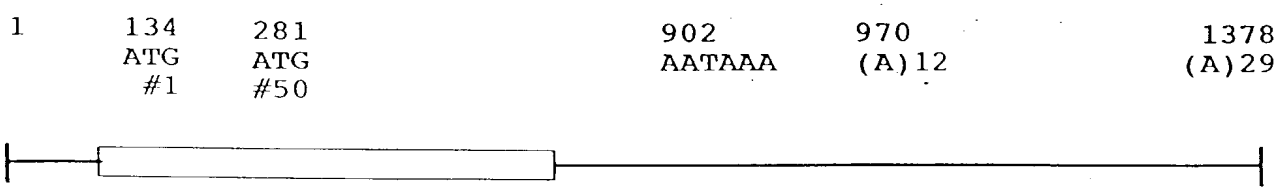
Figures 4A-D (Amended)

Recombination-Associated Protein cDNA Clone

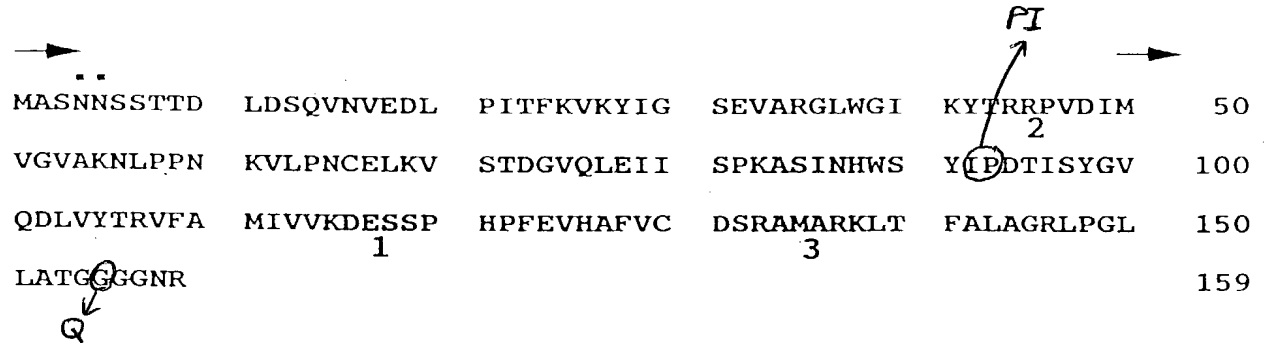
10/031893

A.

DRAP: 1378 bp Eco RI insert
ORF 104-610



B.



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Potential	D1-------(30 to 70)---D2---(35±)---E1	Motifs
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D.

Rad 51	Mouse - Human	L L I V - D - S
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RecA	E. Coli	V I V V - D - S
Drosophila DRAP		M I V V K D E S S P
FLP Recombinase		M I A L K D E T N P
T4 Gene 32 Protein		I L V V K D P A A P
		M I A V - DVE M G E
		K - GFS S E
Human Topoisomerase I		I K D E - - P
		K D G S S E
		GFS S P

Figures 4A-D (Mark-up)

10031893 .U71902

Customer No.: 07278

PATENT TRADEMARK OFFICE

Docket No.: 2567/1F496US2

DECLARATION
AND POWER OF ATTORNEY
Original Application

As a below named inventor, I declare that the information given herein is true, that I believe that I am the original, first and sole inventor if only one name is listed at 1 below, or a joint inventor if plural inventors are named below, of the invention entitled:

DROSPHILA RECOMBINATION-ASSOCIATED PROTEIN AND METHODS FOR USE

which is described and claimed in:

[] the attached specification or

[X] the specification in application Serial No. 10/031,893, filed January 22, 2002
(for declaration not accompanying appl.)

that I do not know and do not believe that the same was ever known or used in the United States of America before my or our invention thereof or patented or described in any printed publication in any country before my or our invention thereof, or more than one year prior to this application, or in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to this application, that I acknowledge my duty to disclose information of which I am aware which is material to patentability in accordance with 37 CFR §1.56. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I hereby claim the priority benefits under 35 U.S.C. §119 of any application(s) for patent or inventor's certificate listed below. All foreign applications for patent or inventor's certificate on this invention filed by me or my legal representatives or assigns prior to the application(s) of which priority is claimed are also identified below.

PRIOR APPLICATION(S), IF ANY, OF WHICH PRIORITY IS CLAIMED

COUNTRY	APPLICATION NO.	DATE OF FILING
U.S.	60/144,736	July 21, 1999
U.S.	09/621,377	July 21, 2000

ALL FOREIGN APPLICATIONS, IF ANY, FILED PRIOR TO THE APPLICATION(S) OF WHICH PRIORITY IS CLAIMED

COUNTRY	APPLICATION NO.	DATE OF FILING
PCT	PCT/US00/19901	July 21, 2000

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney(s) and/or agents(s) to prosecute this application and transact all business in the Patent and Trademark office connected therewith: Gordon D. Coplein #19,165, Michael J. Sweedler #19,937, S. Peter Ludwig #25,351, Paul Fields #20,298, Marc S. Gross #19,614, Joseph B. Lerch #26,936, Melvin C. Garner #26,272, Ethan Horwitz #27,646, Adda C. Gogoris #29,714, Bert J. Lewen #19,407, Henry Sternberg #22,408, Peter C. Schechter #31,662, Robert Schaffer #31,194, Robert C. Sullivan, Jr. #30,499, Ira J. Levy #35,587, Joseph R. Robinson #33,448, Scott G. Lindvall #40,325, Paul F. Fehlner, Ph.D. #35,135, David Leason #36,195, Mitchell Bernstein, Ph.D. #46,550.

all of the firm of DARBY & DARBY P.C., 805 Third Avenue, New York, NY 10022

SEND CORRESPONDENCE TO:

DARBY & DARBY P.C.
Post Office Box 5257
New York, NY 10150-5257

DIRECT TELEPHONE CALLS TO:

Mitchell Bernstein, Ph.D.

212-527-7700

FULL NAME AND RESIDENCE OF INVENTOR 1

100

LAST NAME: Eisen FIRST NAME: Andrew MIDDLE NAME:

CITY: Rockville STATE OR FOREIGN COUNTRY: MD COUNTRY OF CITIZENSHIP: U.S.

POST OFFICE ADDRESS: 12412 St. James Road MD CITY: Rockville STATE OR COUNTRY: MD ZIP CODE: 20850

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR : _____ DATED: 6/23/02

Andrew Eisen